MODERN DIAGNOSIS OF EPSTEIN-BARR VIRUS INFECTIONS AND POST-TRANSPLANT LYMPHOPROLIFERATIVE DISEASE

by

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Academic Dissertation

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2. ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals.

I Aalto SM, Linnavuori K, Peltola H, Vuori E, Weissbrich B, Schubert J, Hedman L, Hedman K. Immunoreactivation of Epstein-Barr virus due to cytomegalovirus primary infection. J Med Virol 56:186-191, 1998

II Mattila PS, Aalto SM, Heikkilä L, Mattila S, Nieminen M, Auvinen E, Hedman K, Tarkkanen J. Malignancies after heart transplantation: presence of Epstein-Barr virus and cytomegalovirus. Clin Transplant 15:337-342, 2001

III Aalto SM, Juvonen E, Tarkkanen J, Volin L, Ruutu T, Mattila PS, Piiparinen H, Knuutila S, Hedman K. Lymphoproliferative disease after allogeneic stem cell transplantation – pre-emptive diagnosis by quantification of Epstein-Barr virus DNA in serum. J Clin Virol 28:275-283, 2003

IV Juvonen E, Aalto SM, Tarkkanen J, Volin L, Mattila PS, Knuutila S, Ruutu T, Hedman K. High incidence of PTLD after non-T-cell-depleted allogeneic haematopoietic stem cell transplantation as a consequence of intensive immunosuppressive treatment. Bone Marrow Transplant 32:97-102, 2003

V Loginov R, Aalto S, Piiparinen H, Halme L, Arola J, Hedman K, Höckerstedt K, Lautenschlager I**.** Monitoring of EBV-DNAemia by quantitative real-time PCR after adult liver transplantation**.** J Clin Virol 37:104-108, 2006

VI Aalto SM, Juvonen E, Tarkkanen J, Volin L, Haario H, Ruutu T, Hedman K. Molecular diagnosis and prediction of EBV infection after allogeneic stem cell transplantation. A manuscript, submitted for publication.

3. ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ALL	acute lymphoblastic leukemia
ALT	alanine aminotransferase
AML	acute myeloid leukemia
ATG	anti-thymocyte globulin
AZA	azathioprine
BL	Burkitt's lymphoma
CAEBV	chronic active EBV infection
CML	chronic myeloid leukemia
CMP	cardiomyopathy
CMV	cytomegalovirus
CP	chronic phase
CR	complete remission
CRP	C-reactive protein
CTL	cytotoxic T lymphocyte
CyA	cyclosporin A
DLI	donor lymphocyte infusion
EA	early antigen
EBER	Epstein-Barr-encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EIA	enzyme immunoassay
EM	electron microscopy
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GVHD	graft-versus-host disease
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7
HIV	human immunodeficiency virus
HD	Hodgkin's lymphoma
HPV	human papilloma virus
IFA	immunofluorescence assay

IHD	ischemic heart disease
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
LCL	lymphoblastoid cell line
LMP	latent membrane protein
MDS RAEB	myelodysplastic syndrome, refractory anaemia with excess of blasts
MP	methylprednisolone
MTX	methotrexate
NHL	non-Hodgkin's lymphoma
NPC	nasopharyngeal carcinoma
PBS	phosphate buffered saline
PBST	phosphate buffered saline-Tween
PCR	polymerase chain reaction
PNA	peptide nucleic acid
PTLD	post-transplant lymphoproliferative disorder
QPCR	real-time quantitative polymerase chain reaction
RNA	ribonucleic acid
SCT	stem cell transplantation
SOT	solid organ transplantation
TBI	total body irradiation
UNCT	undifferentiated carcinoma of nasopharyngeal type
VCA	viral capsid antigen

4. SUMMARY

Serological diagnosis of Epstein-Barr virus (EBV) infections is classically based on detection of heterophile antibodies and on measurement of antibody reactivity for various EBV antigens. Characteristic of EBV primary infection are positive viral capsid antigen (VCA) IgM and IgG and lack of Epstein-Barr virus nuclear antigen (EBNA)-1 antibodies. During convalescence, VCA IgM antibodies usually disappear and the EBNA-1 becomes detectable, while VCA IgG persists for life. Avidity of VCA IgG separates primary and secondary infections both in immunocompetent and immunocompromised individuals. Reactivations of EBV have been encountered among immunocompromised hosts such as organ transplant recipients or patients with human immunodeficiency virus (HIV) infection but also among immunocompetent individuals.

Serological diagnosis of herpes virus infections is hampered by concurrent expression of IgM for heterologous members of this virus family. In serological diagnosis of mononucleosis it is quite common to encounter EBV IgM-positive patients with high-avidity VCA IgG antibodies indicating past immunity. To assess the frequency of such diagnostic findings and to understand their etiology, we used specific EBV IgG, IgM and IgG avidity tests among immunocompetent or immuno-suppressed patients with well-documented cytomegalovirus (CMV) primary infection. Among EBV-seropositive patients with CMV primary infection, a large proportion (23%) showed antibody profiles of EBV reactivation. In contrast, EBV primary infections did not appear to induce immunoreactivation of CMV.

We also wanted to clarify the viral etiology of malignancies occurring after heart transplantation. During the years 1985 and 1999 a total of 249 cardiac transplantations were performed at the Helsinki University Central Hospital. Twenty recipients were diagnosed as having a malignant tumor. We determined the presence of EBV by an in situ hybridisation assay (see below) and manifest CMV infections by immunochemical quantitation. Half of the lymphomas expressed EBV RNA. The majority of the patients with EBV positive lymphomas had a history of post-transplant CMV infection. The result suggests that CMV might have a contributory role in the development of EBV-associated lymphomas.

Epstein-Barr virus associated post-transplant lymphoproliferative disease (PTLD) is a life threatening complication of allogeneic stem cell or solid organ transplantation. Following organ

transplantation the incidence rate of this syndrome varies according to the type of graft and the immunosuppressive regimen. One of our goals was to evaluate the incidence of symptomatic EBV infections after organ transplantation and to improve the diagnosis of PTLD. First, we set up an in situ hybridisation assay for detection of EBV RNA in tissue sections. Second, we set up in 1999 – to our knowledge first in Finland for any microbial pathogen – a quantitative EBV-DNA-PCR (qPCR), and assessed its diagnostic value by using ordinary serum samples.

Diagnosis of PTLD after stem-cell transplantation has been demanding to many reasons: conventional serology is not useful, tissue biopsies are needed for the in situ hybridisation assay, lack of specific clinical signs or symptoms and rapid progression of the disease. We determined the incidence of fatal PTLD among 257 allogenic stem cell transplantations (SCT) performed in Finland during 1994-1999. The patients in studies III and IV are also included in the larger patient material in study VI. The post-morten analysis revealed 18 cases of PTLD. From a subset of the PTLD cases identified (12/18) and a series of corresponding stem-cell recipient controls (36), consecutive samples of serum were studied by EBV qPCR. All the PTLD patients were positive for EBV-DNA with progressively rising copy numbers. In most PTLD patients EBV-DNA became detectable 70 days after SCT or 23 days before death; i.e. earlier than symptoms which appeared 15 days before death. Among SCT control group, EBV-DNA occurred only sporadically and EBV-DNA levels remained relatively low (below $21x10^3$ per ml). We concluded that qPCR for EBV-DNA in serum is a highly sensitive (100%) and specific (96%) diagnostic approach. Intensely immunosuppressed stem-cell recipients are at a great risk of developing PTLD and can now be monitored for EBV-DNA as an indicator for pre-emptive treatment of this life-threatening disease.

Subsequently, the relationship between the occurrence of EBV-associated PTLD and the intensity of immunosuppression was determined. All the patients who developed PTLD had been given anti-thymocyte globulin (ATG) either for treatment of steroid-resistant acute graft-versus-host disease (GVHD) or as a part of conditioning (all patients receiving an unrelated donor transplant). In conclusion, in transplantations from an HLA-identical donor with a non-T-cell depleted graft, the risk of PTLD correlated strongly with the intensity and quality of the immunosuppressive treatment.

Furthermore, we wanted to find out how often and how severely do EBV related complications exist after liver transplantation in Finland; 105 adult liver transplant recipients were retrospectively monitored for EBV infections by EBV qPCR of sequential plasma samples (1284) obtained during the first year after transplantation, and also for CMV, human herpesvirus-6 and -7. EBV DNA was detected in fourteen patients (13%) during the first 12 months; 4% of the plasma specimens contained EBV DNA. Most EBV reactivations occurred concomitantly with the betaherpes viruses. All the EBV DNAemias with low copy number subsided quickly. The peak viral loads of 13 patients were relatively low (below $6.6x10^3$ per ml). No clinical signs or symptoms could be attributed to these EBV-DNAemias. Although most EBV reactivations were harmless, fatal PTLD was diagnosed in one patient. In conclusion, as liver transplantations are concerned, EBV PTLD is a rare but hazardous disease. Also among liver transplant recipients EBV qPCR seems to be essential for the diagnosis of PTLD. During PTLD treatment, follow-up of EBV DNA levels is highly useful for guidance of therapy decisions.

Finally, we wanted to determine the number and clinical significance of EBV infections among a large cohort of allogenic, unselected stem cell transplant (SCT) recipients. Of the 406 SCT performed in Helsinki during 1988-1999, 5479 consecutive samples of serum were retrospectively studied by quantitative EBV PCR, and the clinical data were reviewed. Patients in study III and IV are part of this analysis. Overall, EBV DNA in at least one serum sample was seen in 57 (14.0%) patients, of whom 22 (5.4%) showed progressively rising and ultimately high (>50 000) levels of EBV DNA (median 54 million copies/ml). In addition, 16 (4.0%) patients showed EBV DNA in low copy numbers (median 5370 /ml) shortly before death. Among the SCT survivors, transient EBV-DNAemias (median 6300 /ml), apparently corresponding to asymptomatic EBV infections were seen in 19 (4.7%) patients. Thereby, low-level EBV-DNA positivity in serum occurs relatively frequently after SCT and may subside without specific treatment. However, high molecular copy numbers (>50 000) predict the development of PTLD very strongly, are not spontaneously reversible, and warrant immediate treatment. In molecular diagnosis, the first criterion is the EBV DNA level: low (<50 000 copies/ml) or high (>50 000 copies/ml). If this EBV DNA level is ≥50 000, the patient can be classified as having life-threatening EBV infection. If this initial EBV DNA level is <50 000, the rapidity of the increase in the DNA levels is considered, according to the mean value of the derivatives of the log-transformed EBV DNA values. If this value exceeds 3.6, the patient is at a high risk of having life-threatening EBV infection.

5. INTRODUCTION

Infection by Epstein-Barr virus (EBV) occurs in approximately 95% of the world's adult population. After primary infection, the virus persists lifelong in circulating B-lymphocytes and free virus is intermittently detectable in saliva (Gerber et al., 1972). In developing countries, primary infection usually occurs during the first few years of life and is often asymptomatic. However, in developed populations, primary infection is often delayed until adolescence or adulthood, in many cases producing the characteristic clinical features of infectious mononucleosis, including sore throath, fever, malaise, lymphadenopathy, and mild hepatitis (Henle et al., 1968).

EBV was the first human virus implicated in oncogenesis. EBV has been implicated in the pathogenesis of lymphoid and epithelial malignancies including endemic Burkitt's lymphoma, undifferentiated nasopharyngeal carcinoma, Hodgkin's lymphoma, the immunodeficiency-related non-Hodgkin's lymphomas, post-transplant lymphoproliferative disease (PTLD), some T cell lymphomas, and more recently certain cancers of the stomach and smooth muscle (Burkitt, 1962; zur Hausen et al., 1970; Hanto et al., 1981 and 1982; Strauss et al., 1993; Baumforth et al., 1999). In 1964, Epstein and his co-workers identified herpes-virus like particles by EM in a cell line established from a Burkitt's lymphoma biopsy (Epstein et al., 1964). Subsequently, it was shown that sera from patients with Burkitt's lymphoma had much higher antibody titres to EBV antigens than did controls (Klein et al., 1966; Epstein 1973; Henle et Henle, 1979). The detection of EBV DNA in Burkitt's lymphoma and nasopharyngeal carcinoma tumour cells, and the experimental production in 1973 of lymphomas in cotton top marmosets and owl monkeys exposed to EBV, strongly suggests that this virus has oncogenic potential in both human and non-human primates (Miller et al., 1977). Lehtinen et al. (1993) have reported elevated EBV early antigen (EA) and EBV nuclear antigen (EBNA) antibody levels associated with increased risk of malignant lymphoma. These elevated antibody responses may be due to destruction of neoplastic EBV positive B-cells and/or activation of latent EBV infection early in the lymphomagenesis. Although the EBV genome can be detected in malignant cells, a causative role of EBV in many of these tumors has not been firmly established.

The precise mechanisms by which EBV transforms cells are only now being elucidated. The virus appears to adopt different forms of latent infection in different types of tumors. Specific patterns of latent EBV gene expression are characteristic of the different EBV-associated malignancies.

Polymorphic non-Hodgkins's lymphomas which arise in organ transplant recipients, frequently express the full complement of EBV latent genes (Young et al., 1989). Of these latent genes, latent membrane protein 1 (LMP1) has been shown to act as a constitutively activated receptor and to cause aberrant cellular signalling. This illustrates the complex relation between the virus and the host cell. Another important factor influencing EBV gene expression is the immune response: viral latent proteins to which immunodominant cytotoxic lymphocyte responses are directed, the Epstein-Barr virus nuclear antigen 3 (EBNA3) family of proteins, are downregulated in virus-associated tumors arising in immunocompetent individuals (Murray et al., 2001). Epstein-Barr virus nuclear protein 1 (EBNA1), an essential protein for the maintenance of EBV infection, has evolved to evade immunosurveillance by preventing the protein being processed through the MHC class I pathway (Murray et al., 2001). Studies of the function the EBV latent genes have revealed the ability of these proteins to target specific cell signalling pathways.

The importance of the immune system in suppressing EBV mediated B cell growth and division is underscored by the frequent development of EBV associated lymphoproliferative disease in various immunosuppressive states. The prototypic EBV induced lymphoproliferative disorder arises as a result of the iatrogenic immunosuppression of organ transplant recipients, although similar disorders occur in some of the inherited immunodeficiencies and in patients with acquired immunodeficiency syndrome (AIDS). Lymphoproliferations that arise after iatrogenic immunosuppression for transplant recipients are almost always of B cell origin, and are known as post-transplant lymphoproliferative disease or disorders (PTLD). PTLDs are extremely heterogenous, ranging from polyclonal hyperplasia to aggressive, non-Hodgkin's lymphoma (Hanto et al., 1982; O'Reilly et al., 1997; Faye et al., 2005). PTLD may involve virtually any organ system, including the central nervous system, bone marrow, intestine and lungs.

The association of EBV with malignancy has been characterized by analysis of the terminal repeat sequences in DNA extracts (Raab-Traub et al., 1986). The linear genomes produced during lytic infection carry variable numbers of direct tandem 500bp repeats at each end. When the termini of EBV genome fuse, the number of terminal repeats incorporated is variable and is a property of the resulting circular episome. This number of terminal repeats appears to be stable through the multiple rounds of latent replication during cellular proliferation. The demonstration of a single fused terminal fragment in tumour tissue suggests that the virus has been present through all stages during monoclonal proliferation of tumour cells and supports the etiological role of EBV in tumorigenesis (Raab-Traub et al., 1986).

6. REVIEW OF THE LITERATURE

6.1 HISTORY

In 1958, Denis Burkitt, a British surgeon working in Uganda, described a common cancer affecting children in regions of equatorial Africa. Burkitt wrote and spoke widely about the unusual epidemiologic and clinical features of this lymphoma (Burkitt, 1962, 1963 and 1966). The climatic and geographical distribution of Burkitt's lymphoma, as it came to be known, led Burkitt to suggest that a virus might be responsible. After this, Epstein-Barr virus (EBV) was discovered in 1964 by electron microscopy of cells cultured from Burkitt's lymphoma tissue by Epstein, Achong, and Barr (Epstein et al., 1964). Four years later, EBV was found to be the cause of heterophile-positive infectious mononucleosis (Henle et al., 1968). EBV DNA was detected in tissues from patients with nasopharyngeal carcinoma in 1970 (zur Hausen et al., 1970). Since then, EBV was found to be associated with non-Hodgkin's lymphoma and oral hairy leukoplakia in patients with the acquired immunodeficiency syndrome (AIDS) (Zieglar et al., 1982; Greenspan et al., 1985). A couple of years later, EBV was found to be associated with T-cell lymphomas and Hodgkin's lymphoma (Jones et al., 1988; Mueller al, 1989).

6.2 CLASSIFICATION

Epstein-Barr virus (EBV) belongs to the herpesvirus family. EBV is a member of the genus *Lymphocryptovirus*, which belongs to the gamma herpesvirinae. The gamma herpesvirus subfamily includes both the gamma 1, or *Lymphocryptovirus*, and gamma 2, or *Rhadinovirus*, genera (Kieff et Rickinson, 2001). EBV is the only human *Lymphocryptovirus*, and the Kaposi's sarcoma-associated herpesvirus is the only human *Rhadinovirus* (Chang et al., 1994; Kieff et Rickinson, 2001). Many Old World primate species have their own endemic *Lymphocryptovirus*, and some New World primate species also have endemic *Lymphocryptoviruses* (Kieff et Rickinson, 2001). EBV was the first lymphocryptovirus to be discovered (Epstein et al, 1964). The other *Lymphocryptoviruses* have been named according to their natural host species. The *Lymphocryptoviruses* are structurally similar to each other (in genome structure and gene organization) (Kieff et al., 1991 and 2001).

The gamma herpesvirus subfamily classification was established on the basis of similarity in biologic properties. EBV and Kaposi's sarcoma-associated herpesvirus have particularly limited

host ranges and both are associated with human malignancies (Kieff et Rickinson, 2001). These and other gamma herpesviruses also establish latent infection in lymphocytes, but also beta herpesviruses such as human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and cytomegalovirus (CMV) latently infect lymphocytes. Taxonomists have renamed EBV as human herpesvirus 4 (HHV-4). However, most authors still use the name EBV (Kieff et Rickinson, 2001).

6.3 VIRUS AND GENOME STRUCTURE

Kieff and his collegues have described the structure of EBV (Fundamental Virology, 1991 and Virology 2001): EBV has (1) a toroid-shaped protein core wrapped with DNA (Figure I), (2) a nucleocapsid with 162 capsomeres, (3) a protein tegument between the nucleocapsid and the envelope and (4) an outer envelope with external glycoprotein spikes (Kieff et al., 1991). The major EBV capsid proteins are of 160, 47 and 28kd. The glycoprotein gp350/220 predominates on the outer envelope (Dolyniuk et al., 1976; Thorley-Lawson et al., 1979). The EBV genome is composed of linear, douple-stranded 184-kbp DNA; and regions of the EBV genome have become known by their position on a *Bam*HI restriction endonuclease map of the genome. The characteristic features of EBV and other lymphocryptoviridae include according to Kieff and his collegues (1991 and 2001): (1) a single overall format and gene arrangement (Given et al., 1978; Dambaugh et al, 1980; Raab-Traub et al., 1980), (2) tandemly reiterated 0.5-kbp terminal direct repeats (TRs) (Hayward et al, 1977; Given et al, 1979; Kintner et al. 1979), and (3) tandemly reiterated 3-kbp internal direct repeats (IRls) (Given D et al., 1979; Hayward et al., 1980; Cheung et al., 1981), which divide the genome into two largely unique sequence domains (short and long; U_S and U_L). U_S and U_L have almost all of the genome coding capacity. Although, largely unique DNA, U_S and U_L include perfect and imperfect tandem DNA repeats, most of which are within ORS (Kieff et Rickinson, 2001).

The reiteration frequency of the EBV tandem perfect repeats becomes variable during viral DNA replication, with the average number of repeats being identical to the parent genome (Kieff et Rickinson, 2001). When EBV infects a cell, the genome becomes an episome with a characteristic number of tandem repeats, dependent on the number in the parental genome, with variation during viral DNA replication and the unique cleavage and joining events of the single infecting viral genome (Kieff et Rickinson, 2001). If the infection is nonpermissive for viral replication and permissive for latent infection, each EBV episome in progeny infected cells will have the same number of tandem repeats as the parent cell (Kieff et Rickinson, 2001). The number of tandem

repeats is therefore useful in determining whether a group of latently infected cells arose from a single common progenitor cell or from multiple cells (Brown et al., 1986; Raab-Traub et al., 1986). EBV isolates differ in their tandem reiteration frequency. Individual isolates tend to have a constant number of repeats (Dambaugh et al, 1980; Heller et al., 1981; Raab-Traub et al., 1986; Katz et al, 1988; Kieff et al., 1991). EBV was the first herpesvirus whose genome was completely cloned (Dambaugh et al., 1980; Raab-Traub et al, 1980) and sequenced (Baer et al., 1984).

Figure I. A toroid form.

6.4 THE MAJOR EBV-OUTER ENVELOPE GLYCOPROTEIN, gp350/220

The major EBV outer-envelope glycoprotein, gp350/220, is the EBV ligand for CD21 (Wells et al., 1982; Nemerow et al., 1987; Tanner et al., 1987). Gp350 and gp220 are transplated from an abundant late-replication-cycle EBV mRNA, part of which is spliced in frame to give the mRNA for the smaller, 220kd, glycoprotein (Biggin et al., 1984; Beisel et al., 1985; Whang et al., 1987). CD21 is the only B-lymphocyte surface protein which binds to gp350/220 (Tanner et al., 1987). Soluble gp350/220 can saturate B-lymphocyte receptors and block virus infection, indicating an essential role for the gp350/220-interaction in virus adsorption (Tanner et al., 1988; Nemerow et al., 1989). The peptide EDPGFFNVE, which is contained in gp350/220 and C3d (the complement component which binds to CD21), at least partially mediates the binding of C3d or gp350 to CD21, since synthetic peptides containing this sequence bind to CD21 and block EBV infection (Nemerow et al., 1989; Tanner et al., 1987 and 1988).

EBV gp350/220 not only mediates EBV absorption, but also mediates initial penetration (Tanner et al., 1987). Multiple gp350/220 molecules on the EBV outer envelope cross-link B-lymphocyte plasma membrane CD21, resulting in patching and capping of CD21 and surface immunoglobulin (Tanner et al., 1987). Surface cross-linking of CD21 causes normal B-lymphocyte to enlarge and clump but does not increase cell RNA or DNA synthesis (Tanner et al., 1987). After capping, EBV undergo polar endocytosis into smooth membrane vesicles (Nemerow and Cooper, 1984; Tanner et

al., 1987). The EBV envelope presumably fuses with the vesicle membrane, releasing the nucleocapsids into the cytoplasm (Kieff and Liebowitz, 1991). Gp350/220 may play some role in the EBV envelope vesicle membrane fusion, since gp350 has a domain which could be amphopathic (Beisel et al., 1985; Tanner et al., 1987). However, the EBV gH homolog, gp85, is the second most abundant envelope glycoprotein and probably plays a key role in EBV envelope vesicle membrane fusion (Edson et Thorley-Lawson, 1981 and 1983; Strnad et al., 1983; Heineman et al., 1988). Monoclonal antibodies to gp85 do not affect virus adsorption but do inhibit EBV envelope and cell membrane fusion (Miller and Hutt-Fletcher, 1988). Most EBV neutralizing antibody response is directed to gp350/220 (Thorley-Lawson et Poodry, 1982).

6.5 ESTABLISHMENT OF LATENCY OR INITIATION OF VIRUS REPLICATION

By analogy with other DNA viruses which replicate in the nucleus, the cytoskeleton is likely to mediate EBV capsid transport to the nucleus (Dales and Chardonet, 1973). Genome circularization either precedes or coincides with earliest virus gene expression. Cell transcription factors probably determine if latent or lytic infection ensues. B-lymphocyte-specific transcription factors may be necessary for latent or lytic cycle EBV promoter activity (Farrel et al., 1983).

The usual outcome of B-lymphocyte infection with EBV is persistent latent infection and transformation of cell growth to perpetual proliferation. The effect on cell growth is rapid and efficient (Henderson et al., 1977; Sudgen et Mark, 1977; Mark et Sugden, 1982). There are probably sequential changes in cell growth after initial infection (Moss et al., 1984). EBV induces RNA synthesis, immunoglobulin secretion, expression of several B-cell activation markers, DNA synthesis and cell division (Kintner et Sugden, 1981; Thorley-Lawson et al., 1982; Gordon et al., 1984; Amen et al., 1986). EBV-infected B lymphocytes produce autostimulartory substances which promote cell growth *in vitro* (Gordon et al., 1984). Autocrine growth stimulation may contribute to the proliferation of B lymphocytes latently infected with EBV in vitro as well as to EBV transformation of B lymphocytes in vivo (Kieff et Liebowitz, 1991).

6.5.1 ZEBRA PROTEIN

The switch from the latent to the lytic life cycle of the virus is controlled by two immediate-early proteins: ZEBRA (also called EB1 and Zta), encoded by the BZLF1 gene and Rta, encoded by the BRLF1 gene (Countryman et al., 1985 and 1987; Hardwick et al., 1988; Rooney et al., 1989; Feederle et al., 2000). Both transcription factors are expressed simultaneously following induction

of the lytic cycle (Takada et Ono, 1989; Sinclair et al., 1991; Gradoville et al., 2002). Each protein activates a separate class of EBV lytic cycle genes, and together the two proteins synergize to activate a third class of lytic cycle genes (Ragoczy et Miller, 1999). Some early lytic genes, such as BMRF1, are activated in synergy by ZEBRA and Rta. However, ZEBRA suppresses Rta's ability to activate a late gene, BLRF2. El-Guindy and Miller (2004) showed that this repressive activity is dependent on the phosphorylation state of ZEBRA. ZEBRA plays an indispensable role in driving the lytic cycle of EBV.

ZEBRA is a sequence-specific DNA binding protein that forms a homodimer via its pseudo-leucine-zipper dimerization domain. ZEBRA recognizes canonical AP-1 sites well as a spectrum of 7-bp sequences termed ZEBRA response elements (ZREs) (Lehman et al., 1998). ZEBRA triggers the switch from the latent to the lytic state by binding to ZREs in promoters of viral and cellular genes and activating their transcription (Farrell et al., 1989; Flemington et Speck, 1990). Transcriptional activation of responsive genes by ZEBRA requires that the viral protein interacts with several cellular proteins. The protein directly interacts with TATA binding protein and components of the TFIIA complex via its activation domain. The activation domain of ZEBRA stabilizes the TFIIA-TFIID complex and recruits the TFIIB complex, which further stabilizes the preinitiation complex (Chi et al., 1995). The transcriptional activity of ZEBRA is also dependent on the ability of ZEBRA to recruit CREB binding protein, a transcriptional coactivator that possesses intrinsic histone acetyltransferase activity (Adamson et Kenney, 1999; Zerby et al., 1999).

ZEBRA is also indispensable for lytic viral DNA replication. The protein binds to multiple motifs in *oriLyt*, (Hammerschmidt et Sugden, 1988; Lieberman et Berk, 1990; Schepers et al., 1993). The minimum lytic viral DNA replication machinery in EBV consists of ZEBRA plus six EBV-encoded replication proteins (Fixman et al., 1992). ZEBRA physically interacts with three of these proteins: the viral DNA polymerase (BALF5), the DNA polymerase processivity factor (BMRF1) via its DNA binding domain, and the helicase-primase complex (BBLF4-BSLF1:BBLF2/3) via its activation domain (Zhang et al., 1996; Gao et al., 1998; Baumann et al., 1999). The latter interactions suggest that ZEBRA might recruit and stabilize the replisome on *oriLyt.*

6.6 LYTIC INFECTION

6.6.1 EARLY LYTIC INFECTION

Lytic replication differs from the latent amplification in that multiple rounds of replication are initiated within *oriLyt* and the replication process is dependent on EBV-encoded proteins (Hammerschmidt et Sugden, 1988; Fixman et al., 1995; Tsurami et al., 2005). A two-stage model has been proposed for the *oriLyt*-mediated EBV DNA replication (Schepers et al., 1996). Essential EBV replication genes were identified by Fixman and colleagues (1995) and this allowed identification of seven essential core EBV replication genes (BZLF1, BALF5, BMRF, BMRF1, BALF2, BBLF4, BSLF1, BBLF2/3). These genes are necessary for *oriLyt*-specific DNA replication (Fixman et al., 1995; Tsurami et al., 2005).

At least 30 EBV mRNAs are early mRNAs and almost 30 EBV mRNAs are late mRNAs (Hummel et Kieff, 1982; Bankier et al., 1983; Baer et al., 1984; Hudson et al., 1985; Gibson et al., 1986; Sample et al., 1986; Biggin et al., 1987). Early and late mRNAs are intermingled through most of the EBV genome (Kieff et Liebowitz, 1991). Frequently, different promoters initiate nested transcripts which begin with different open-reading frames and terminate at the same poyadenylation site so that the longest mRNA includes all of the shorter mRNAs and reading frames 3' to the primary reading frame. Some early and late genes are spliced whereas others are not (Kieff et Liebowitz, 1991). The function of quite many EBV genes was first guessed from comparison of predicted amino acid sequences of other herpesvirus proteins of known function (Kieff et Liebowitz, 1991).

Among the EBV early genes identified by their homology to early genes of other herpesviruses are several which are linked to DNA replication. These include DNA polymerase (BALF5), major DNA-binding protein (BALF2), ribonucleotide reductase (BORF2 and BARF1), thymidine kinase (BXLF1), and alkaline exonuclease (GLF5) (Hummel et Kieff, 1982; Nikas et al., 1986; Kieff et Liebowitz, 1991; Tsurumi et al., 1998). These genes are distributed through the long EBV US domain (Kieff et Liebowitz, 1991). The EBV DNA polymerase has been extensiviely purified and is 117kd in size (Roubal et al., 1981; Kallin et al., 1985). Partially purified DNA polymerase was associated with several other EBV nuclear proteins, including the 50-kd protein encoded by BMRF1 (Li et al., 1987; Kieff et Rickinson, 2001; Johannsen et al., 2004). The EBV, varizella zoster virus (VZV) and herpes simplex virus (HSV) ribonucleotide reductases have extensively

homology (Gibson et al., 1984). The large subunit of EBV ribonucleotide reductase is 85kd and is confined to multiple, discrete regions in the cytoplasm of productively infected cells (Nikas et al., 1986; Goldschmidts et al., 1987). The large subunit is a delayed early protein which accumulates in cells approximately 4hr after the BMRF1 nuclear early protein (Pearson et al., 1983; Goldschmidts et al., 1987). Methanol fixation of cells destroys the immunologic reactivity of the large subunit, suggesting that it may be a major component of the restricted early antigen complex (Kieff et Liebowitz, 1991).

In addition to the two immediate-early proteins BZLF1 and BRFL1, two EBV early proteins – BSMLF1 and BMRF1 – may *trans*-activate other early EBV genes (Kieff et Liebowitz, 1991; Kieff et Rickinson, 2001). Both are quite abundant early nuclear proteins (Pearson et al., 1983; Cho et al., 1985; Wong et Levine, 1989; Fixman et al., 1995). BSLMF1 is a *trans*-activator of gene expression which acts synergistically with BZLF1 or BRLF1 in inducing higher-level expression in transient expression assays (Lieberman et al., 1986; Wong et Levine, 1986; Oguro et al., 1987; Chavier et al., 1989; Chevallier et al., 1986 and 1989; Kieff et Liebowitz, 1991). BSMLF1 increases cytoplasmic transport of unspliced mRNAs (Ruvolo et al., 1998; Boyle et al., 1999). BSMLF1 has been suggested to be a trans-activator of translation. Soon after induction of the lytic cycle, viral DNA is amplified to yield monomeric plasmid progeny DNA. The BZLF1 protein acts as an immediate-early *trans*-activator and also as an *oriLyt*-binding protein (Schepers et al., 1996; Tsurumi et al., 2005).

One abundant EBV early protein is the 135-kd, single-stranded DNA-binding protein, BALF2 (Hummel et Kieff, 1982). This protein has primary amino acid sequence homology to the major HSV DNA-binding protein, ICP8, which appears to function in DNA replication (Kieff et Liebowitz, 1991; Kieff et Rickinson, 2001). A second RNA that is less abundant encodes the 18-kd protein BHRF1, which has extensive homology with Bcl-2 (Austin et al., 1988; Pearson et al., 1987; Henderson et al., 1993; Kieff et Rickinson, 2001).

The three proteins encoded by the BBLF4, BSLF1 and BBLF2/3 genes form a tight complex and are predicted to act as helicase, primase and helicase-primase associated proteins, respectively, have sequence homology to the herpes simplex virus type 1 UL5, UL52 and UL8 genes (Fixman et al., 1995; Tsurami et al., 2005). It has been suggested that all products of the EBV replication genes (BALF5, BMRF1, BALF2, BBLF4, BSLF1 and BBLF2/3, except for the BZLF1 protein) work together at replication forks to synthesise leading and lagging strands of the EBV genome (Tsurumi,

2001; Tsumi et al., 2005). EBV DNA replication genes are dependent on expression of the BZLF1, BRLF1, and BSMLF1 transactivators (Kieff et Rickinson, 2001). EBV DNA replication also requires topoisomerase 1 and 2 activity (Kawanishi, 1993). The redistribution of BZLF1 and BMRF1 gene products to the same nuclear site may identify the site at which viral DNA is replicated (Takaki et al., 1984; Kieff et Rickinson, 2001).

6.6.2 VIRAL DNA REPLICATION

EBV DNA replication appears to be biphasic and a two-stage model has been proposed (Pfuller et Hammerschmidt, 1996). With induction of lytic EBV replication, the episome copy number increases, suggesting that circular episomal DNA replication is a precursor to subsequent DNA replication (Shaw, 1985). The BZLF1 protein binds to the *oriLyt*. In the late phase of the viral productive cycle, the EBV genome is amplified 100- to 1000-fold (Tsurumi et al., 2005).

Essential EBV replication genes are BZLF1, BALF5, BMRF1, BALF2, BBLF4, BSLF1, BBLF2/3. The BZLF1 protein functions as an *oriLyt*-binding protein and an immediate-early transactivator (Schepers et al., 1996; Tsurumi et al., 2005). The product of the BZLF1 gene is also known as Zta, ZEBRA and EB1. Expression of BZLF1 alone is sufficient to trigger the entire lytic cascade (Countryman et Miller, 1985; Rooney et al., 1989; Amon et Farrell, 2005). The BALF5 gene encodes the DNA Pol catalytic subunit and the BMRF1 gene encodes the DNA Pol accessory subunit (Tsurumi et al., 1993, 1994 and 2005). A single-stranded DNA-binding protein is encoded by the BALF2 gene (Tsurumi et al., 1997 and 1998). The three proteins encoded by the BBLF4, BSLF1 and BBLF2/3 genes form a complex and are predicted to act as helicase, primase and helicase-primase associated proteins, respectively (Yokoyama et al., 1999; Fujii et al., 2000). All except for the BZLF1 protein work together at replication fork (Tsurumi, 2001). The specific interactions between the six viral replication proteins are essential for EBV DNA replication.

Two EBV DNA segments (DL and DR left and right duplications of DNA with *oriLyt* activity) which have strong early promoter activity function as origins of lytic-infection viral DNA replication (Hammerschmidt et Sugden, 1988). Most likely initiation of the lytic EBV DNA replication involves the formation of an initiation complex, which consists of two essential upstream and downstream domains. The former contains several BZLF1 binding sites, and the latter includes binding sites for several cellular proteins (Tsurumi et al., 2005). The first step would be the binding of the BZFL1 protein and two transcription factors, ZBP-89 and Sp1, to *oriLyt* to form an initial complex (Tsurumi et al., 2005). ZBP-89 and Sp1 are known to stimulate replication

(Baumann et al., 1999). The interaction of the BZLF1 protein with the BBLF4/BSLF1/BBLF2/3 complex is in line with its recruitment of the viral helicase-primase complex to *oriLyt* (Gao et al., 1998; Tsurumi et al., 2005). Also, BZLF1 protein can interact with both BALF5 and BMRF1 proteins (Zhang et al., 1996; Baumann et al., 1999). The BALF2 ssDNA-binding protein then interacts with the BZLF1-BBLF4/BSLF1/BBLF2/3 complex (Gao et al., 1998).These proteins together would have the potential to open up the duplex DNA in the origin region and synthesise RNA primers (Tsurumi et al., 2005). The interaction between the EBV *Pol* holoenzyme and the BBLF4/BSLF1/FFLF2/3 complex may be crucially important in initiation of DNA synthesis (Fujii et al., 2000; Tsurumi et al., 2005).

The BALF5 *Pol* catalytic and BMRF1 *Pol* subunits function as the *Pol* holoenzyme, presumably synthesising both leading and lagging strands (Tsurumi et al., 2005). The BALF2 protein binds ssDNA templates and facilitates movement of the EBV DNA *Pol* holoenzyme on the ssDNA template. The EBV helicase and primase complex may then bind to the lagging strand at the fork, translocate in the 5'-to-3' direction and synthesise the RNA primer (Tsurumi et al., 2005). The BALF5 *Pol* catalytic subunit interacts with each component of the BBLF4/BSLF1/BBLF2/3 complex (Fujii et al., 2000). Thus, the six viral replication proteins appear to all work at the replication fork as the replication machinery (Tsurami et al., 2005).

EBV productive DNA replication occurs at discrete sites in nuclei, called replication compartments (Tsurumi et al., 2005). BZLF1 *oriLyt* binding proteins show a diffuse pattern of distribution in the nuclei in immediate early stages of induction and then become associated with the replicating EBV genome with granular spots in the replication compartments throughout the phase for lytic infection (Tsurumi, et al., 2005). Almost all abundantly expressed BMRF1 proteins bind to DNA indicating that they not only act at viral replication forks as a Pol processive factor, but are also widely distributed on newly synthesised EBV genomic DNA (Tsurumi et al., 2005).

6.6.3 LATE LYTIC INFECTION

Conserved herpesvirus genes are known to encode five capsid proteins, five envelope proteins, and 10 tegument proteins (Gompels et al., 1988; Davison et Taylor, 1987). Accordingly, EBV BcLF1, BDLF1, BFRF3, BORF1, and BBRF1 ORFs are likely to encode the major, minor, and smallest capsid proteins (MCP, mCP, and sCP, respectively), mCP-binding protein (mCPBP) and the portal protein (Johannsen et al., 2004). The antibodies against viral capsid antigen (VCA) complex are the important serodiagnostic markers of EBV infection. Then, the tegument proteins are encoded by the EBV BPLF1, BOLF1, BVRF1, BGLF1, BGLF4, BGLF2, BBRF2, BSRF1, BGLF3, and BBLF1 ORFs (Hummel et Kieff, 1982; Vroman et al., 1985; Russo et al., 1996; Davison et al., 2002; de Jesus et al., 2003; Johannsen et al., 2004). EBV BNRF1 and BLRF2 probably encode γ-herpesvirus-unique tegument proteins (Hummel et Kieff, 1982; Baer et al., 1984; Cameron et al., 1987; Serio et al., 1996; Johannsen et al., 2004). The EBV homologues of gB (BALF4), gH (BXLF2), and gL (BKRF2) have been detected by specific antibodies in EB virions. Furthermore, EBV BLLF1, BZLF2, and BDLF3 ORF-specific antibodies have detected EBV-unique gp350, gp42, and gp150 (Hutt-Fletcher et Lake, 2001; Johannsen et al., 2004). EBV BMRF2-encoded protein may also be found in virus envelopes because an RGD (arginine-glycine-aspartic acid) motif that may function as a ligand for an integrin receptor (Tugizov et al., 2003).

The known EBV glycoprotein genes are BLLF1 (gp350/220) BALF4 (gB or gp110), BXLF2 (gH or gp85), BKRF2, BZLF2 (gp42), BILF2 (gp55/80 or gp78), BDLF3 (gp150), BLFR1 (gp15), BBRF3 (gp84/113) and BILF1 (gp64) (Biggin et al., 1984; Hummel et al., 1984; Beisel et al., 1985; Gong et al., 1987; Heineman et al., 1988; Oba et al., 1988; Mackett et al., 1990; Nuebling et Mueller-Lantzsch, 1991; Hutt-Fletcher et al., 2001; Kieff et Rickinson, 2001; Johannsen et al., 2004). At least two other EBV genes, BHRF1 and BMRF2, probably encode glycoproteins. BILF2, in a recombinant vacciniavirus expression system, was expressed as a glycoprotein named as gp78 or gp55/80 (Mackett et al., 1990; Kieff et Rickinson, 2001; Johannsen et al., 2004). The BILF2 glycoprotein corresponds to an abundant glycoprotein in purified virus preparantions (Kieff et Rickinson, 2001). However, antibody to BILF2 fails to neutralize virus infectivity (Mackett et al., 1990). BDLF3 cDNA analysis confirms that BDLF3 mRNA is not spliced (Heineman, 1987). BDLF3 (gp150) is a highly glycosylated protein of only 26-kd expressed late in EBV replication (Heineman, 1987; Johannsen et al., 2004). BALF4 is collinearly homologus to HSV-1 glycoprotein B (gB), which is a major virion and infected cell-surface glycoprotein (Pellet et al., 1985; Emini et al., 1987; Gong et al., 1987). BALF4 encodes two late mRNAs. The BALF4 3-kb mRNA translates

a 93-kd protein, EBV glycoprotein B (gB or gp110), which is glycosylated to 110 kd (Gong et al., 1987 and 1990). Mature EB virions have a small amount of full-length EBV gB (gp110) and, which is one of the most abundant late EBV proteins (Hutt-Fletcher et Lake, 2001). The intracellular distribution of EBV gB (gp110) closely parallels that of an endoplasmic reticulum resident protein (Gong et Kieff, 1990). Light or electron microscopy localizes EBV gB (gp110) to the inner and outer nuclear membrane and to cytoplasmic membranes frequently surrounding enveloped virus. Despite EBV gB (gp110)s presence in the inner nuclear membrane through which nucleocapsids bud to acquire their initial envelope, EBV gB (gp110) cannot be demonstrated in enveloped intracellular or extracellular virus (Gong et al., 1987 and 1990). Thus EBV gB (gp110) is a very abundant viral glycoprotein present in the cell nucleus and in the endoplasmic reticulum, but is not a major structural protein of the virion. EBV gB appears to be the critical component for EBV glycoprotein-mediated cell fusion (McShane et Longnecker, 2004).

In contrast to EBV gB (gp110), two other EBV glycoproteins EBV gH (gp85) and gp350/220, are found on the virus and in the plasma membrane of lytically infected cells (Dolyniuk et al., 1976; Strnad et al., 1979; Thorley-Lawson, 1979; Thorley-Lawson et al., 1979, 1980 and 1982; Hoffman et al., 1980; Mueller-Lantzsch et al., 1980; North et al., 1980; Morgan et al., 1983 and 1984; Hummel et al., 1984; Beisel et al., 1985; Heineman et al., 1988; Oba et al., 1988; Gong et al., 1990; Johannsen et al., 2004). EBV gH (gp85) is encoded by BXLF2, which has significant collinear homology to HSV-1 gH (McGeoch et Davison, 1986; Heineman, 1987; Oba et al., 1988), whereas gp350/220 is encoded by BLLF, which has only a small region of distant homology to HSV-1 gC (Beisel et al., 1985; Tanner et al., 1987). EBV gH (gp85) is a relatively minor virus component which appears to be important in fusion between virus and cell membranes (Miller et Hutt-Fletcher, 1988), whereas gp350/220 is the dominant external virus glycoprotein which mediates virus binding to the B-lymphocyte receptor, CD21 (see above). EBV gH (gp85) expressed in NIH 3T3 cells localizes to the internal cytoplasmic and nuclear membranes, whereas gp350/220 localizes to the Golgi apparatus and plasma membrane (Heineman et al., 1987; Whang et al., 1987) .This suggests that EBV gH (gp85) may require another virus protein to bring it to the plasma membrane of lytically infected lymphocytes, where it is characteristically found, whereas gp350/220 appears to possess the signals necessary for efficient intracellular transport, Golgi processing, and plasma-membrane insertion. Gp350/220 is not only the most abundant viral protein in the lytically infected cell plasma membrane, but it is also one of the most abundant late viral proteins and the most abundant protein on the outer surface of the virus (Kieff et Liebowitz, 1991). Most of the human EBV neutralizing antibodies and significant immune T cell reactivity are directed against

gp350/220 (Thorley-Lawson et Poodry, 1982; Ulaeto et al., 1988). Gp350/220 is therefore an essential component of any prospective EBV vaccine.

Early EM observations of lymphocytes lytically infected with EBV revealed the presence of nucleocapsids in the cytoplasm (Kieff et Rickinson, 2001). It has been hypothesised that the virus probably acquires an initial envelope as it buds through the nuclear membrane, that de-envelopment occurs within cytoplasmic vesicles resulting in release of nucleocapsids into the cytoplasm (Kieff et Rickinson, 2001). Re-envelopment occurs at the plasma membrane, where the virus acquires a definitive envelope rich in gp350/220 and gH/gL. Late in infection, as the cell deteriorates, partially enveloped or initially enveloped virus may be released in substantial quantity (Kieff et Rickinson, 2001). Overview of EBV lytic cycle is presented in Figure II.

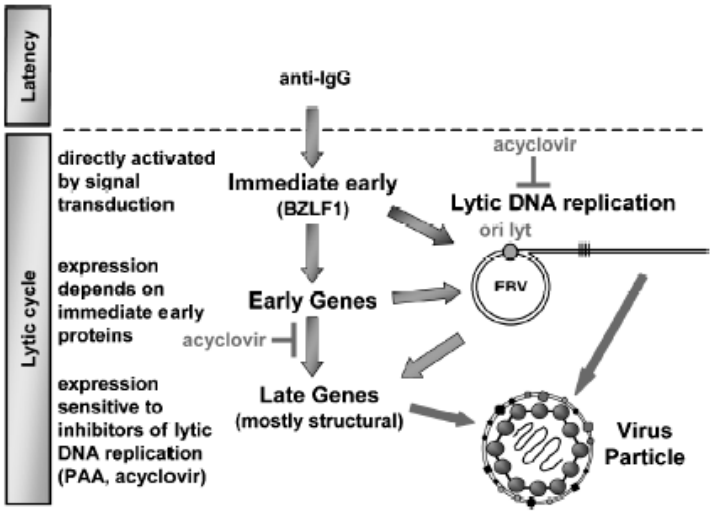

Figure II. Overview of EBV lytic cycle presented by Amon and Farrell (Rev Med Virol 15:149-156, 2005). The lytic cycle can be induced with anti-IgG, which cross-links the B cell receptor to mimic antigen binding. Immediate early, early and late genes are expressed in sequential order. Late gene expression is prevented by inhibitors of lytic DNA replication such as acyclovir.

6.7 EBV TYPES: EBV1 AND EBV2 (EBV A AND B)

Two EBV types, EBV-1 and EBV-2 (or type A and B, respectively) have been identified in most human populations (Bornkamm et al., 1980; Dambaugh et al., 1980; Heller et al., 1981; Dambaugh et al., 1984; Zimber et al., 1986; Young et al., 1987; Rowe et al., 1989). These were initially defined

on the basis or divergence within the coding region for EBV-determined nuclear antigen 2 (EBNA-2), a viral gene expressed during latent infection that is crucial for B-cell immortalization (Rabson et al., 1982; Dambaugh et al., 1984; Adldinger et al., 1985; Skare et al., 1985; Hammerschmidt et Sugden, 1989). The major differences between EBV-1 and EBV-2 genomes are in the latent infection cycle genes EBNA-2 (Dambaugh et al., 1984; Adldinger et al., 1985), EBNA leader protein (EBNA-LP or also known as EBNA5) (Dambaugh et al., 1984; Sample J et al., 1986), EBNA-3A, -3B, and -3C (also known as EBNA-3, EBNA-4 or EBNA-6) (Rowe et al., 1989) and in the EBERs (Arrand et al., 1989). Further distinction between EBV isolates can be made on the basis of mutations affecting different regions of the genome (Walling et al., 1994; Walling et Raab-Traub, 1994; Gratama et Ernberg, 1995).

The two families vary in their geographic distribution, tissue tropism, and biologic behaviour. Type 1 EBV is ubiquitous, while type 2 is commonly isolated only in parts of Africa endemic for malaria and Burkitt's lymphoma (Zimber et al., 1986; Young et al., 1987). EBV-1 can infect oropharyngeal epithelial cells and peripheral blood lymphocytes, while type 2, when found in healthy Westerners, is seen in the oropharynx and only rarely in the peripheral blood (Sixbey et al., 1989; Sculley et al., 1988 and 1990). EBV-1 efficiently immortalizes B cells, while EBV-2 immortalizes B cells far less effectively in vitro, and these B cells are less hardy in culture than those infected by type 1 (Rickinson et al., 1987).

Many studies have shown an increased incidence of oropharyngeal and peripheral blood infection by EBV-2 in various immunocompromised populations. A higher rate of EBV-2 infection is found in equatorial Africa, and this may be related to the generalized depression in T-cell function and intense polyclonal B-cell stimulation commonly seen in this part of the world (Sixbey et al., 1989). In the West, patients debilitated by age and illness have been found to harbour EBV-2 (Boyle et al., 1993). Also, an increased incidence of EBV-2 has been shown in patients with acquired immunodeficiency syndrome (AIDS) (Sculley et al., 1988; Sixbey et al., 1989) EBV-2 infection also appears to be more common in immunocompromised patients with some lymphoid neoplasms, such as AIDS-associated non-Hodgkin's lymphomas (Boyle et al., 1991; Borisch et al., 1992). Interestingly, Buissom and colleagues (1994) reported changes in the dominant EBV type during human immunodeficiency virus (HIV) infection: EBNA-2 serotyping and genotyping showed that HIV-infected patients were highly infected by EBV-2, and that the dominant strain was mostly retained. However, during a follow-up study, a change in the dominant viral strain was observed in two patients (Buissom et al., 1994). It has been shown that up to one third of cardiac transplant

patients carry EBV type 2 in their peripheral blood, a much higher figure than the 3% observed in the general population (Sculley et al., 1990; Kyaw et al., 1992). However, despite the immunocompromised state of the hosts, solid-organ transplant recipients with post-transplant lymphoproliferative disorders (PTLD) frequently harbored EBV type 1 (Frank et al., 1995).

Falk and colleagues (1997) reported direct identification of EBV types and virants in clinical samples by PCR. Both types and multiple variants of each type were found with a much higher frequency from saliva samples of HIV-carrying individuals compared with healthy individuals or immunocompetent patients with infectious mononucleosis: 74% HIV-1 positive individuals secreted EBV in saliva, of these 48% were positive for EBV type 1, 23% were positive for both type 1 and type 2 EBV, and 29% were positive for type 2 alone (Falk et al., 1997). Both type 1 and 2 were detected in the B-lymphocytes from one healthy individual, and one individual had two variants of type 1 EBV (Falk et al., 1997). Type 1 EBV alone was detected in mouthwash samples from six patients with infectious mononucleosis (Falk et al., 1997). It seems that an intact immune response to the virus prevents superinfection by exogenous virus. Superinfection is probably a regular threat, because the virus is ubiquitous, and symptom-free virus carriers frequently secrete biologically active virus (Falk et al., 1997). Nevertheless, multiple virus variants have been identified among isolates from approximately 15% of healthy carriers (Gratama et al., 1994). In one case, 14 different variants were identified in the same individual. In some cases, these variants were the result of changes in only one EBNA-protein. Falk and colleagues (1997) suggested that these variants arise because of recombination between endogenous EBV genomes during latency, or more likely, during lytic multiplication in the oropharynx. Superinfection appears to be rare in healthy donors. Interestingly, a recent study by Tierney and colleagues (2006) shows that patients with infectious mononucleosis frequently harbour different EBV strains. Tierney and colleagues (2006) studied blood and throat wash samples from 14 patients with infectious mononucleosis; most patients carried two or more EBV type 1 strains, one patient carried 2 EBV type 2 strains, and one patient carried both virus types.

In contrast, for immunosuppressed individuals, several studies have shown dual carriage of type 1 and type 2, as well as the presence of multiple variants (Gratama et Ernberg, 1995). In cases where the molecular weights of most EBNAs varied, it is likely that the presence of several variants in the same patient is due to superinfection because of breakdown of immunological barriers (Falk et al., 1997). In immunosuppressed patients, this may also relate to increased replication of endogenous EBV because of a less tightly controlled immunoregulation of latency. This in turn may speed up

generation of new variants by recombination between endogenous and superinfecting viral genomes (Falk et al., 1997).

The complete EBV2 genome, for strain AG876, was sequenced by Dolan and colleagues (2006). Comparison with the published EBV1 sequence demonstrated that the two sequences are collinear and, outside the known diverged alleles, generally very close (Dolan et al., 2006). The EBNA1 gene was identified as another diverged locus, although its variation is believed not to correlate with EBV type. Overall, genomic comparisons indicated that the two EBV types should be considered as belonging to the same virus species (Dolan et al., 2006).

6.8 EBV INFECTION

Infection of humans with EBV usually occurs by contact with oral secretions. Oropharyngeal epithelium is permissive for virus replication (Sixbey et al., 1983 and 1984). Nearly all seropositive persons intermitently shed the virus in saliva (Gerber et al., 1972; Yao et al., 1985). Because two studies have reported EBV in male and female genital secretions, direct spread of virus during sexual intercourse is possible (Sixbey et al., 1986; Israele et al., 1991). Crawford and colleagues (2002) found a highly significant association between sexual intercourse and EBV seropositivity, with correlation with increasing numbers of sex partners. These data suggest that sexual contact, or a factor closely associated with it, is an important factor in acquisition of EBV during the teenage years (Crawford et al., 2002). However, it is very difficult to distinguish between this and transmission of virus by kissing or orogenital contact during sexual intercourse, and therefore, an indirect association remains a possibility (Crawford et al., 2002). Recently, Thomas and colleagues (2006) investigated the risk of sexual transmission; low levels of virus detected in genital secretions compared to saliva suggest that this is not a major transmission route. There is a four to six weeks' incubation period before the clinical symptoms of infectious mononucleosis appear (Rickinson et Kieff, 2001).

Earlier studies indicated that the virus replicates in epithelial cells in the oropharynx (Sixbey et al., 1984), and B lymphocytes are infected at early stage of disease (Allday et al., 1988). Other studies have suggested that B cells in the oropharynx may be the primary site of infection (Anagnostopoulos et al., 1995; Niedobitek et al., 1997) (Figure III). Histologic studies could not identify any viral infection of tonsillar epithelium but did detect small numbers of lytically infected

B-lymphoid cells, usually near the crypt epithelium (Weiss et Mohared, 1989; Anagnostopoulos et al., 1995; Rickinson et Kieff 2001).

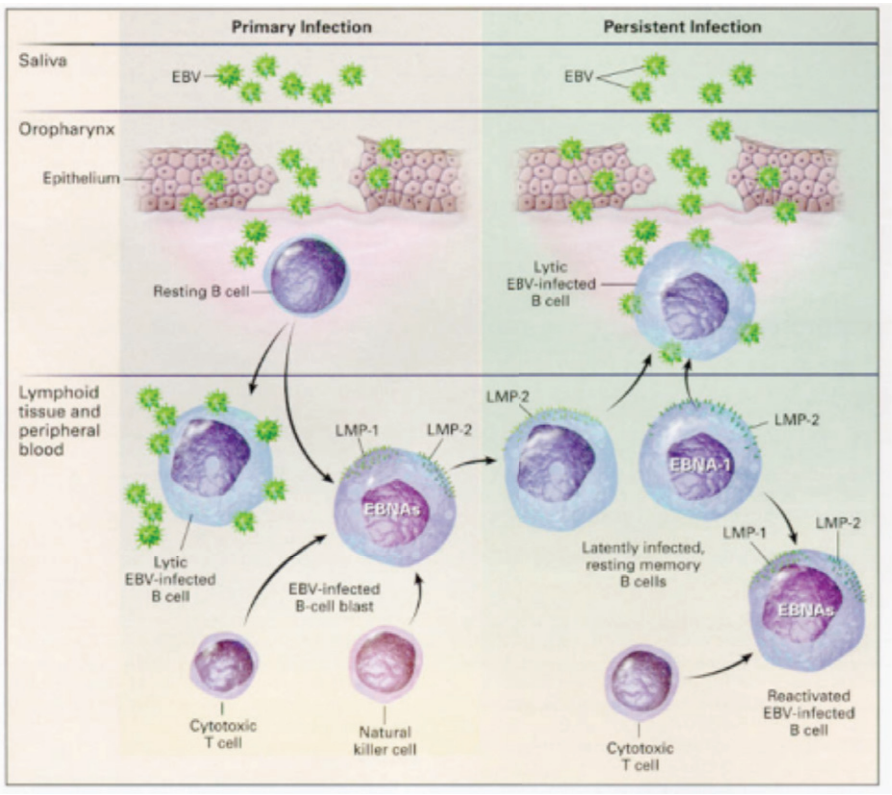

Figure III (Cohen, NEJM 343:481-492, 2000). Model of EBV infections in humans. In the oropharynx, EBV directly infects B cells or epithelial cells, which in turn infect B cells. During primary infection, the EBV-infected B cells undergo a lytic cycle with production of virus, or express the full complement of latent viral proteins. The infection is limited by natural killer cells and cytotoxic T cells. Beyond convalescence, EBV is present in the peripheral blood in latently infected memory B cells that express latent membrane protein (LMP) 2 and possibly EBV nuclear antigen (EBNA) 1. The EBV carrying memory B cells can undergo EBV reactivation. Some latently infected cells undergo lytic replication in the oropharynx, resulting in shedding of virus into the saliva or infection of epithelial cells with release of virus. Reproduced with permission.

6.9 LATENT INFECTION

Early in the course of primary infection, EBV establishes latent infection in B lymphocytes. These resting memory B cells are the site of EBV persistence within the body (Babcock et al., 1998). Once infected, the B cells carry the viral genome in their nuclei. The viral genome of non-replicating EBV exists in closed circles, called episomes. The observation that EBV can be eradicated in bone

marrow-transplant recipients who have received therapy that ablates their hematopoietic cells, but not their oropharyngeal cells, provides further evidence that B cells are the site of EBV persistence (Gratama et al., 1988). In normal adults, from one to 50 B cells per million are latently infected with EBV, and the number of these latently infected cells remains stable over years (Birx et al., 1986; Babcock et al., 1998).

EBV enters B cells by interaction of the major viral glycoprotein gp350/220 with the complement receptor (CR2/CD21), which is the receptor for complement component C3d. The viral glycoproteins gp25 (gL) and gp42/38 that form a complex with viral gp85 (gH). This complex mediates an interaction between EBV and major histocompatibility complex (MHC) class II molecules, which serve as a co-receptor for virus entry into B cells (Knox et al., 1995; Murray et al., 2001). Crosslinking of CD 21 activates B lymphocytes and causes some immediate effects: tyrosine kinase (Lck) activation and calcium mobilisation, increased mRNA synthesis, homotypic cell adhesion, blast transformation, surface CD 23 expression and interleukin 6 production (Murray et al., 2001). Once the viral genome has been uncoated and transferred to the nucleus, circularisation and transcription from the Wp promoter begin a cascade of events leading to expression of all the latent genes. The EBV nuclear antigen (EBNA) leader protein (EBNA-LP) and EBNA2 are the first proteins to be detected and these are sufficient to advance the cells to early G1 phase (Murray et al., 2001). The proliferation of EBV infected cells depends on high cell density and on the autocrine production of cytokines. Later, EBV^{+} cells are less dependent upon autocrine growth mechanisms. Following that, the virus establishes a latent infection in memory B cells, and this is characterised by limited expression of virus latent genes (Murray et al., 2001).

It was believed earlier, that EBV exists in the peripheral blood within the IgD^{-} memory B-cell pool, (Babcock et al., 1998). However, Ehlin-Henriksson and colleagues (2003) have shown that B-lymphocyte subpopulations are equally susceptible to Epstein-Barr virus infection, irrespective of immunoglobulin isotype expression. EBV gene expression in these cells is restricted to latent membrane protein 2A (LMP2A), EBERs, BamHI A RNAs and in some cases EBNA1 (Tierney, et al., 1994; Miyashita et al., 1997; Babcock et al., 1998; Chen et al., 1999). This narrowing of viral gene expression after primary infection is consistent with the reversion of infected cells from an activated lymphoblast to a resting memory B-cell phenotype (Rickinson et Kieff, 2001). It has been suggested that the most EBV-infected B-cells in the blood are EBNA1 and LMP2A mRNA negative and these transcripts might be detectable only in such cells that have recently left the lymphoid tissue environment (Babcock et al., 1999; Rickinson et Kieff, 2001). This implies the

existence of a circulating pool of EBV-positive cells in which all viral antigen expression is limited to the EBERs and possibly the BamHI A RNAs (Rickinson et Kieff, 2001).

Analysis of lymphoid tissues suggests that the proportion of B cells that carry the virus is very low, comparable to levels in the blood (Anagnostopoulos, 1995; Babcock et al., 1998; Rickinson et Kieff, 2001). It has been reported that in some cases EBER-positive centroblasts, EBNA2-negative, but occasionally expressing LMP1, are detectable within germinal centres (Araujo, et al., 1999). This is consistent with RT-PCR analysis that has detected LMP1, LMP2A, and EBNA1 mRNAs both in the germinal center B cell and in the memory B cell compartments of tonsillar lymphocytes (Babcock et al., 2000). These represent reactivations from the pre-existing memory cell pool rather than *de novo* infections because such transcripts were consistently present in tonsils without evidence of virus replication (Rickinson et Kieff, 2001). A subset of healthy tonsils contain EBV^+ naive B cells and these cells express the latency III programme and show an activated phenotype (Joseph et al., 2000). These cells presumably are either eliminated by virus-specific cytotoxic T lymphocytes (CTLs) or differentiate to memory B cells, which then leave the tonsil. Some of these memory B cells will pass through mucosal lymphoid tissue and terminally differentiate into plasma cells, whereupon they might enter the lytic cycle (Murray et al., 2001). Interestingly, latency III infection was found only in tonsils in which virus replication was occurring to provide a source of progeny virus for *de novo* infections (Babcock et al., 2000). It seems that reactivations from the latently infected memory B-cells and *de novo* infections may be occurring simultaneously in the lymphoid tissues of virus carriers (Rickinson et Kieff, 2001).

6.10 EBV INFECTION OF EPITHELIAL CELLS

Epithelial cells generally do not express CD21, suggesting that EBV enters these tissue cells by other cellular receptors. Various human epithelial cells can be infected in vitro either by direct contact with high-titre virus supernatant or by mixed culture with EBV producing cells. Sixbey and colleagues (1983) have shown in vitro evidence for direct infection by EBV and replication of the virus in cultured normal human epithelial cells. In vivo epithelial cells might be infected by virtue of their close proximity to lytically infected B cells resident near or within epithelial tissues (Murray et al., 2001). Virus made by epithelial cells lacking MHC class II (E-EBV) contains increased levels of gp42 and is more infectious for B cells compared with virus made in B cells (B-EBV) (Murray et al., 2001). B-EBV was equally infectious for epithelial cells than was E-EBV. These data suggest that primary EBV infection of epithelial tissues could lead to the production of

virus with a particular tropism for B cells. Virus produced by B cells near to epithelia could more readily infect epithelial tissues with subsequent release of virus into saliva (Murray et al., 2001).

EBV is not usually detectable in normal epithelial tissues, including desquamated oropharyngeal cells and tonsillar epithelium from infectious mononucleosis patients (Niedobitek et al., 2000), and normal epithelium adjacent to EBV^+ undifferentiated nasopharyngeal carcinoma (Sam et al., 1993) and gastric carcinomas (Gulley et al., 1996). Nevertheless, the virus can be detected in pre-invasive undifferentiated nasopharyngeal carcinoma (Pathmanathan et al., 1995) and dysplastic gastric epithelium (Gulley et al., 1996), suggesting that EBV might be an early trigger in the pathogenesis of these lesions. EBV can also be detected in oral hairy leukoplakia, a benign lesion of the oral epithelia characterised by intense lytic infection (De Souza et al., 1989).

6.11 EBV LATENT PROTEINS

Four types of latent gene expression have been described. In healthy individuals, the virus persists episomally in resting memory B cells. Of the ~100 viral proteins, only LMP-2 is expressed. In addition, the small polyadenylated viral RNAs designated as EBERs 1 and 2 are also produced. This type of latency has been designated type 0 (Thompson et al., 2004). The other three types of latency are characteristic for a heterogenous group of malignancies. Latency I, II, and III are based on patterns of expression of the EBV genome (Thompson et al., 2004).

When peripheral blood lymphocytes from chronic virus carriers are cultured, the few EBV positive B cells give rise to spontaneous outgrowths of EBV-transformed immortalised cell lines, known as lymphoblastoid cell lines (LCLs) (Rickinson et al., 1984). Every cell in an LCL carries multiple copies of circular extrachromosomal viral DNA (episomes) and produces several latent proteins, including six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and three latent membrane proteins (LMPs 1,2A and 2B) (Kieff, 1996). Transcripts from the *Bam*HI A region are also detected, although whether these encode proteins remains controversial (Brooks et al., 1993). LCLs also show abundant expression of the small non-polyadenylated RNAs EBERs 1 and 2. They contribute to oncogenesis by confering resistence to apoptosis and causing induction of interleukin-10, which acts as an autocrine growth (Nanbo et Takada, 2002). EBERs serve as excellent targets for detection of EBV in tumours (Murray et al., 2001). This pattern of latent EBV gene expression is referred to as Latency III (Lat III) and it is characteristic of LCLs, most post-transplant lymphomas and AIDS-related proliferative disorders (Niedobitek et al., 1997; Murray et al., 2001).

Latency I is characteristic of Burkitt's lymphoma (Sbih-Lammali et al., 1996). In Latency I only EBNA1, the EBERs and the BamA transcripts are regularly produced. Latency II is observed in EBV-associated Hodgkin's lymphoma, T-cell non-Hodgkin's lymphoma and undifferentiated nasopharyngeal carcinoma (Cesarman et al., 1999; Murray et al., 2001). In Latency II the EBERs, EBNA1 and BamA are expressed together with LMP1 and LMP2.

The Latency III pattern of EBV gene expression seen in lymphoblastoid cell lines (LCLs) is associated with high-level expression of the B-cell activation markers CD23, CD30, CD39 and CD 70 and of the cellular adhesion molecules leukocyte-function-associate molecule 1 (LFA-1; CD11a/18), LFA-3 (CD 58) and intercellular adhesion molecule 1 (ICAM-1; CD54) (Roowe et al., 1987). These markers are also expressed in high levels when the cells are activated into short-term growth by antigenic or mitogenic stimulation, suggesting that EBV-induced immortalisation can be elicited through the same cellular pathways that drive physiological B-cell proliferation. The ability of EBNA2, EBNA3C and LMP1 to induce LCL-like changes implicates these viral proteins as key factors of the immortalisation process (Wang et al, 1987). The expression of EBV latency factors is shown in Table I.

Expression of EBV latency factors in disease

Latency pattern	EBNA-1	EBNA-2	EBNA-3	LMP-1	LMP-2	EBER	Disease
Type 1	+	-	-	-	-	+	Burkitt's lymphoma
Type 2	+	-	-	+	+	+	Nasopharyng . ca, Hodgkin 's disease T-cell lymphoma
Type 3	+	+	+	+	+	+	Mononucleosis PTLD, XLP, (LCL)
Type 0	+/-	-	-	-	+	+	Healthy carrier

Table I. Expression of EBV latency factors in diseases associated with EBV.

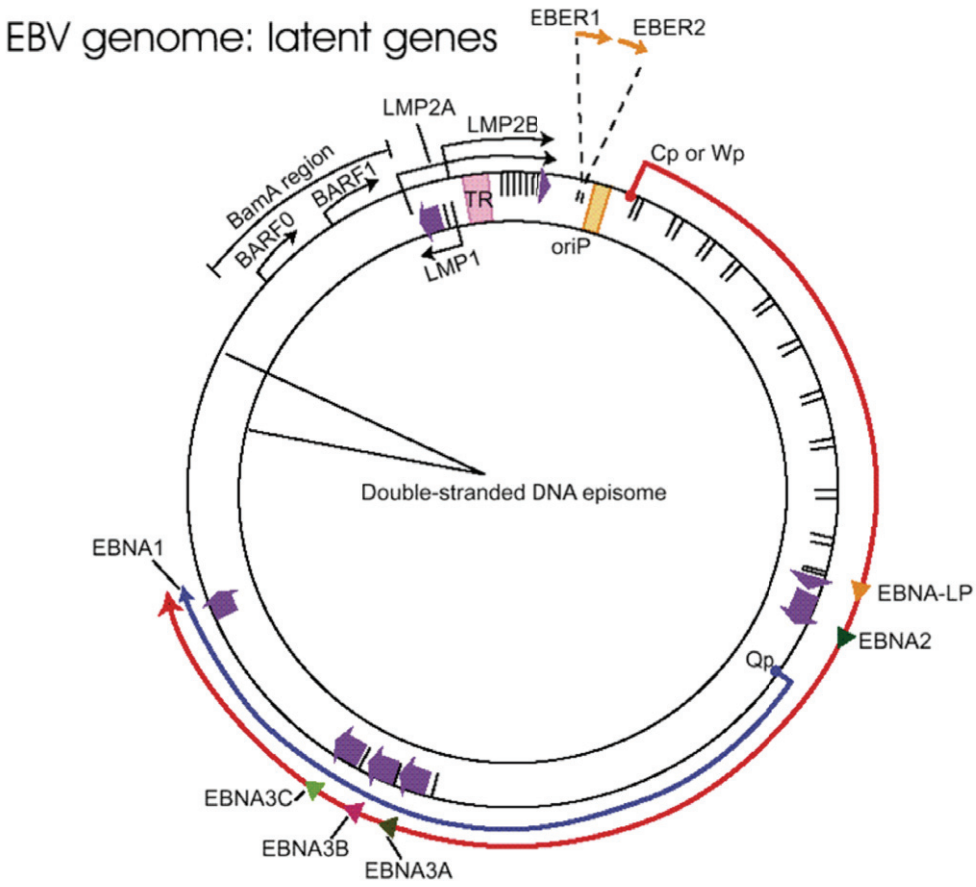

Figure IV. The Epstein-Barr virus (EBV) genome presented by Murray and Young (2001). Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The origin of plasmid replication (oriP) is shown in orange. The large solid blocks (in purple) represent coding exons for each of the latent proteins and the arrows indicate the direction in which they are transcribed: the latent proteins include the six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMPs 1, 2A, 2B). EBNA-LP is transcribed from variable numbers of repetitive exons. LMP2A and LMP2B are composed of multiple exons located either side of the terminal repeat (TR) region, which is formed during the circularisation of the linear DNA to produce the viral episome. The orange arrows at the top represent the highly transcribed non-polyadenylated RNAs EBER1 and EBER2. The outer long arrowed red line represents EBV transcription during a form of latency known as latency III, where all the EBNAs are transcribed from either Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long primary transcript. The inner shorter arrowed blue line represents the EBNA1 transcript originating from the Qp promoter during Lat I and Lat II. Transcripts form the BamA region can be detected during latent infection, but no protein arising from this region has been definitively identified. Shown here are the locations of the BARF0 and BARF1 coding regions. Reproduced with permission.

6.12 FUNCTION OF THE EBV LATENCY PRODUCTS

EBNA1

EBV transforms normal human B-cells into continuously growing lymphoblastoid cell lines (Henle et al., 1967). All transformed cells express the EBV-determined nuclear antigen, EBNA, detected by anticomplement immunofluorescence (Reedman et Klein, 1973). EBNA1 was identified by immunoblotting and radioimmunoelectrophoresis in four EBV-positive but not in three EBV negative cell lines, utilizing four EBV-positive human sera in comparison with two EBV-negative human sera (Strnad et al., 1981). The production of sequence specific antisera conclusively showed that the *Bam*HI K fragment encodes a polypeptide that varies in size between 70 and 92 kd in different EBV-carrying cell lines (Hennessy et Kieff, 1983; Dillner et al., 1984).

EBNA1 is a DNA-binding protein that is required for the replication and maintenance of the episomal EBV genome (Yates et al., 1985). The plasmid origin of viral replication is dependent on the binding of EBNA1 to oriP (Yates et al, 1985). EBNA1 regulates negatively its own expression (Nonkwelo et al., 1996). EBNA1 acts as a transcriptional transactivator and upregulates Cp and the LMP1 promoter (Kieff, 1996). The EBNA1 protein contains a glycine-glycine-alanine (Gly-Gly-Ala) repeat sequence, which varies in size in different EBV isolates (Kieff, 1996). This domain is a cis-acting inhibitor of MHC, class I-restricted presentation and appears to function by inhibiting antigen processing via the ubiquitin-proteasome pathway (Levitskaya et al., 1995). EBNA1 might also have a direct role in oncogenesis (Wilson et al., 1996).

EBNA2

In the immunoblotting study of EBNA1 was detected an additional polypeptide of 81K (Strnad et al., 1981; Hennesy et Kieff, 1983). This protein was further characterized by immunofluorescence and designed as EBNA-2 (Dillner et al., 1985). EBNA-2 was found to be associated with the BamHI WYH region of EBV DNA (Dillner et al., 1985). EBNA2 protein is crucial for the transformation process (Ernberg, 1986). EBNA 2 is a transcriptional activator of both cellular and viral genes, and upregulates the expression of certain B-cell antigens: CD21, CD12, as well as LMP1 and LMP2 (Wang et al., 1987; Kieff, 1996). EBNA2 interacts with a ubiquitous DNA-binding protein RBP-Jκ and this is partly responsible for targeting EBNA2 to promoters (Grossman et al., 1994). The RBP-Jκ homologue in *Drosophila* is involved in signal transduction from the Notch receptor; a pathway that is important in cell fate determination in *Drosophila* and has been

implicated in the development of T cell tumours in humans (Artavanis-Tsakonas et al., 1995). EBNA2 can functionally replace the intracellular region of Notch (Sakai et al., 1998). The *c-myc* oncogene is also a transcriptional target of EBNA2 – important for EBV-induced B-cell proliferation (Kaiser et al., 1999).

EBNA3 family

Dillner and colleagues reported (1986) the existence of a third antigen of the EBNA complex, designated as EBNA-3 (also known as EBNA3A). EBNA3A (EBNA-3) and EBNA3C (also known as EBNA-6) are essential for B-cell transformation in vitro (Robertson, 1997). In was shown that four virally determined nuclear antigens were expressed in EBV transformed cells and the fourth antigen was designated as EBNA-4 (also known as EBNA3B) (Kallin et al., 1985). EBNA3B is a transformation-associated EBV nuclear antigen that has been shown to contain multiple HLA-A11-restricted epitopes with different immunogenicities. A high prevalence of EBNA3B (EBNA-4) mutations has been proposed as mechanism of escaping the CTL response in certain HLA types (Gavioli et al., 1992 and 1993; de Campos-Lima et al., 1993). EBNA3B (EBNA-4) induces expression of vimentin and CD40 (Silins et al., 1994). EBNA3C (EBNA-6) can induce the upregulation of both cellular (CD21) and viral (LMP1) gene expression (Allday et al, 1994), and represses the Cp promoter (Radkov et al., 1997). EBNA3C (EBNA-6) might interact with the retinoblastoma protein, pRB, to promote transformation (Parker et al., 1996). EBNA2 and EBNA3 proteins work together to precisely controlling RBP-Jκ activity, thereby regulating the expression of cellular and viral promoters (Robertson ES, 1997).

EBNA-LP (EBNA-5)

EBNA leader protein or EBNA-LP (also known as EBNA-5) is encoded by the leader of each of the EBNA mRNAs and encodes a protein of variable size depending on the number of *Bam*HI W repeats contained by a particular EBV isolate (Dillner et al., 1986; Wang et al., 1987). EBNA-LP (EBNA-5) is required for the efficient outgrowth of LCLs (Allan et al., 1994). Transient transfection of EBNA-LP (EBNA-5) and EBNA2 into primary B cells induces G0 to G1 transition (Sinclair et al., 1994). EBNA-LP (EBNA-5) can cooperate with EBNA2 in upregulating transcriptional targets of EBNA2, including LMP1 (Nitsche et al., 1997). EBNA-LP (EBNA-5) is a nuclear phosphoprotein, tightly associated with the nuclear matrix and often localized to distinct nuclear bodies (Petti et al., 1990; Kitay et al., 1996). In vitro biochemical studies have demonstrated an interaction of EBNA-LP (EBNA-5) with both retinoblastoma protein (pRb) and p53 (Szekely et al., 1993). It was recently shown that, EBNA-LP, but not other EBNAs, interacts with the p14ARF-

protein, a regulator of the p53 pathway and antagonizes p14ARF-induced cell death (Kashuba et al., 2003).

LMP1

The mRNA coding for the latent membrane protein (LMP) is the most abundant EBV-specific mRNA in latently infected cells. A monospecific anti-LMP serum was produced by Hennessy and colleagues (1984) and the LMP was identified on immunoblots. EBV encodes two integral membrane protein**s,** latent membrane protein 1 and 2 (LMP1 and LMP2). LMP1 acts as an oncogene, and expression of this protein in transgenic mice results in B-cell lymphomas (Wang et al., 1985; Kulwichit et al., 1998). LMP1 induces a signaling response in cells that mimics a constitutively active form of the B-cell-surface-molecule CD40 (Uchida et al., 1999).

At least four signalling pathways, NF-κB, JNK/AP-1, p38/MAPK and JAK/STAT, have been identified in the function of LMP1 (Huen et al., 1995; Eliopoulos et al., 1998 and 1999; Gires et al., 1999; Murray et al., 2001). Within the C-terminus of LMP1 are at least two activating regions, CTAR1 and CTAR2. CTAR1 is essential for EBV-mediated transformation of primary B cells. CTAR2 is required for long-term growth of EBV positive B cells (Izumi et al., 1997; Murray et al., 2001). LMP1 activates JNK cascade via CTAR2 alone (Eliopoulos et al., 1999) whereas activation of the p38/MAPK pathway is mediated by both CTAR1 and CTAR2 (Eliopoulos et al., 1999; Murray et al., 2001). Aggregation of LMP1 within the plasma membrane is a critical for signalling (Gires et al., 1997). LMP1's interaction with the TRAF molecules is important for association of LMP1 with the cytoskeleton (Higuchi et al., 2001; Murray et al., 2001).

The cloning and sequencing of the gene encoding LMP1 from EBV isolates from Chinese or Taiwanese cases of undifferentiated nasopharyngeal carcinoma revealed several mutations (Hu et al., 1991). These so-called de1LMP1 variants show increased tumorgenicity in vitro (Li et al., 1996). This de1LMP1 variant can also be detected in some T-cell lymphomas, Hodgkin's lymphomas, patients with infectious mononucleosis and lymphoblastoide cell lines from healthy controls. Healthy virus carriers have been found to have a similar frequency of these mutations as the virus-infected tumour patients from the same geographical region (Khanim et al., 1996; Murray et al. 2001).

LMP2

The gene encoding LMP2 yields two distinct proteins: LMP2A and LMP2B. The structures of these proteins are similar: both have 12 transmembrane domains and a 27 amino acid cytoplasmic C-terminus (Murray et al., 2001). LMP2A has also a 119 amino acid cytoplasmic N-terminal domain. LMP2A aggregates in patches within the plasma membrane of latently infected B cells (Longnecker et al., 1990). Neither LMP2A nor LMP2B is essential for B-cell transformation (Fruehling et al., 1997; Longnecker, 2000). The LMP2A N-terminal domain contains eight tyrosine residues, two of which (Y74 and Y85) form an immunoreceptor tyrosine-based activation motif (ITAM) (Fuehling et al., 1997). When phosphorylated, the ITAM present in the B-cell receptor mediates lymphocyte proliferation and differentiation (Murray et al., 2001). LMP2A interacts with these protein tyrosine kinases through its phosphorylated ITAM and this association negatively regulates protein tyrosine kinase activity (Fruehling et al., 1997). LMP2A ITAM is responsible for blocking B-cell receptor-stimulated Ca^{2+} mobilisation, tyrosine phosphorylation and activation of EBV lytic cycle in B cells (Miller et al., 1995; Murray et al., 2001).

Expression of LMP2A in the B cells of transgenic mice abrogates normal B-cell development, allowing immunoglobulin-negative cells to colonise peripheral lymphoid organs (Caldwell et al., 1998; Murray et al., 2001). This suggests that LMP2A can drive the proliferation and survival of B cells in the absence of signalling through the B-cell receptor (Caldwell et al., 1998). These data support a role of LMP2 in modifying the normal programme of B-cell development to favour the maintenance of EBV latency and to prevent inappropriate activation of the EBV lytic cycle (Caldwell et al., 1998; Murray et al., 2001). A modulatory role for LMP2B in regulating LMP2A function has been suggested (Scholle et al., 1999). The consistent expression of LMP2A in Hodgkin's lymphoma and nasopharyngeal carcinoma suggests an important function for this protein in oncogenesis but this remains to be shown (Murray et al., 2001). LMP2 also recruits Nedd4-like ubiquitin protein ligases; this might contribute to a block in B-cell signalling (Ikeda et al., 2000). Moreover, LMP2A can transform epithelial cells (Scholle et al., 2000).

EBV-ENCODED RNAS 1 AND 2 (EBERS 1 AND 2)

EBV-encoded RNAs 1 and 2 (EBERs 1 and 2) are nonpolyadenylated, uncapped, noncoding RNAs of 167 and 172 nucleotides, respectively (Baumforth et al., 1999; Thompson et al., 2004). They are expressed abundantly (10^4-10^5 copies per cell) in nearly all EBV-infected cells (Arrand et Rumo, 1982; Howe et al., 1986 and 1989). Oral hairy leukoplakia and some hepatocellular carcinomas are the exceptions; EBERs are not expressed (Gilligan et al., 1990; Niedobitek et al., 1991, Sugawara et al., 1999). EBERs 1 and 2 (in addition to the two LMPs) are expressed in all forms of latency (Murray et al., 2002). EBERs have been implicated in the induction of autocrine growth factors, especially interleukin (IL)-10, and in maintaining the malignant phenotype of Burkitt's lymphoma cells, all of which supports a potential role for these RNAs in oncogenesis (Komano et al., 1999 and 2001; Nanbo et Takada, 2002; Thompson et al., 2004). Furhermore, it has been demonstrated that EBERs confer resistance to interferon (IFN)-α-induced apoptosis by inhibition of douple-stranded (ds) RNA-activated protein kinase (PRK), which is the key mediator of the antiviral effect of IFN-α (Nanbo et Takada, 2002).

6.13 EBV EARLY ANTIGENS (EAs)

During the productive cycle the EBV genome must be linear, and an opening of the episome initiates disruption of latency. Thereafter a few immediate early proteins take part in transactivation of the viral genome (Croen, 1981; Kieff et Liebowitz, 1991). After this, the viral enzymes which are necessary for DNA-replication appear (Kieff et Liebowitz, 1991). These enzymes probably comprise the EBV early antigens (EA). EA is a plethora of immediate-early and early viral proteins, including the BZLF1 immediate early and the abundantly expressed BALF2, BHRF1, BMRF1, and BMLF1 early proteins (Rickinson et Kieff, 2001). On the basis of the localisation in the cell, the EA antigens are divided into two components (Henle et al., 1971). The diffuse antigen (EA-D) is present in both the nucleus and cytoplasm of the infected cell. The restricted component (EA-R) is confined to the cytoplasm, and disappears if the cells are fixed in alcohol (Miller, 1990; Kieff et Liebowitz, 1991; Linde et al., 1992).

EA can be induced in cells which are permissive for EBV replication, and in non-permissive cells. In permissive cells, the induction is followed by replication of EBV DNA and synthesis of structural proteins for assembly of virus progeny (Kieff et Liebowitz, 1991). The host cell provided the facilities necessary for replication, while the virus directs the DNA and protein synthesis of the

cell towards its own purposes. The host cell protein synthesis is not shut down and the cell does not die immediately. However, the initiation of the EBV productive cycle causes the cell to die eventually by the changes induced by EBV, or by the attack from the immune system (Lince et al., 1992). The components of the mature EBV are assembled in the nucleus of the infected cell. The virus probably attains its envelope from the nuclear membrane. Eventually, mature viruses bud out from the cell.

6.14 IMMUNE RESPONSE TO EBV

Infection with EBV results in both humoral and cellular immune responses. During EBV primary infection, three antibodies (-IgG, -IgM and -IgA) are produced against EBV viral capsid antigen (VCA), two antibodies (-IgG and -IgA) are produced in response to early antigen D (EA-D), one antibody (-IgG) in response to early antigen R (EA-R) and three antibodies (-IgG, -IgM and -IgA) to membrane antigen (MA) (Linde, 1996; Rickinson et Kieff, 2001). Each of these antigens is a composite of several distinct viral proteins. EBNA is a complex of six distinct nulear proteins, EBNA1, 2, 3A, 3B, 3C, and –LP, while antibodies to EBNA1 are most frequently detected by the conventional anti-EBNA assay (Rickinson et Kieff, 2001). EA is a complex of immediate-early and early viral proteins (BZLF1, BALF2, BHRF1, BMRF1 and BMLF1 proteins) (Rickinson et Kieff, 2001). Much of VCA reactivity is diffuse cytoplamic fluorescence against virus-encoded nucleocapsid components (BcLF1, BFRF3, BLRF2, and the glycoprotein gp110), which are expressed in late lytically infected cells (Rickinson et Kieff, 2001). Most MA reactivity is directed against gp 350, the most abundant viral protein on the surface of lytically infected cells and on the viral envelope (Rickinson et Kieff, 2001). Anti-MA levels in serum correlate with virus neutralazing activity (Rickinson et Kieff, 2001).

First, during infectious mononucleosis appear IgM, IgG and IgA antibodies to VCA expressed in lytically-infected cells. These antibodies are almost always present in relatively high titres (Linde, 1992). Antibodies to EA-D are found in around 80% of infectious mononucleosis cases. The serological reactivity against the two dominant EBNA proteins, EBNA1 and EBNA2, changes with time after EBV primary infection (Linde, 1992). Antibodies to EBNA2 appear before EBNA1 antibodies. Up to one year after the primary infection, the EBNA1:EBNA2 titre ratio may be <1, but therafter this ratio is normally >1 (Miller et al., 1985; Henle et al., 1987). The VCA IgG antibodies are detected throughout life in healthy persons carrying EBV. IgM antibodies to VCA disappear within one to three months, and EA-D antibodies are replaced by EA-R antibodies

(Linde, 1992). EA-R antibodies reach their maximum between three and six months after EBV primary infection, and may persist in low titres in healthy individuals. Functionally important are antibodies to MA, which appear during the lytic cycle, but these antibodies are seldom measured routinely (Neel et al., 1984). These virus neutralizing antibodies are of low titer and predominantly of IgM class at first, while IgG titers rising relatively late (Rickinson et Kieff, 2001). A transient IgA response to MA is also detectable (Sixbey et Yao, 1992). Antibody responses to the latent membrane proteins (LMP1 or LMP2) have not been seen during infectious mononucleosis but these antibodies are only detectable in a small proportion of healthy carriers with the most sensitive assays (Frech et al., 1990; Meij et al., 1999; Rickinson et Kieff, 2001).

Virus-neutralizing antibodies (anti-MA antibodies) are capable to prevent generalized spreading of the virus (Rickinson et Kieff, 2001). However, the cellular immune response is more important than the humoral for restricting EBV infection. Natural killer cells and CD4+ and CD8+ cytotoxic T cells control EBV-infected B cells during primary infection (Rickinson et al., 1997). In infectious mononucleosis, up to 40 percent of CD8+ T cells are targeted towards one replicative EBV protein sequence, whereas two percent are targeted to one latent EBV protein sequence (Callan et al., 1998). After recovery, HLA-restricted cytotoxic T cells are important in controlling EBV latency, and CD8+ T cells are equally targeted towards replicative and latent antigens (Tan et al., 1999). EBV induces a strong cytotoxic T lymphocyte (CTL) response to multiple antigens. However, a great deal of the cytotoxic-T-cell response is targeted towards the EBNA-3 proteins (Cohen, 2000).

EBV may escape immune surveillance in vivo in several ways (Chu et al., 1999): 1) by altering the amino acid sequence within epitopes recognized by virus specific cytotoxic T lymphocytes (CTLs); 2) via down-regulation of the peptide transporters, thus restricting the loading of MHC class I molecules with peptides derived from intracellular antigens (Niedermann et al., 1995); 3) via down-regulation of CTL immunodominant EBV proteins of EBNA3A, EBNA3B, EBNA3C (EBNA-3, -4, and -6) as in some EBV-associated malignancies, such as Hodgkin's lymphoma (Pallesan et al., 1991; Deacon et al., 1993; Grasser et al., 1994) or, 4) via strong CTL response by certain dominant HLA alleles, such as B8, which mounts a strong response to an EBNA-3 epitope (Burrows et al., 1994) or B27, which mounts a strong response to an EBNA-6 epitope (Brooks et al., 1993) or A11, which mounts a strong response to an EBNA-4 epitope (Gavioli et al., 1993). EBNA3B (EBNA-4) is a transformation-associated EBV nuclear antigen that has been shown to contain multiple HLA-A11-restricted epitopes with different immunogenicities (Chu et al., 1999). A high prevalence of EBNA3B (EBNA-4) mutations has been proposed as a mechanism of escaping the CTL response in

certain HLA types (Gavioli et al., 1992 and 1993; de Campos-Lima, 1993; Chu et al., 1999). Epidemiological studies have shown that mutation of the antigenically determined epitope of EBNA3B (EBNA-4) may play an important role in the development of EBV-associated malignancies (Chu et al., 1999). EBV encodes several important proteins that show sequence and functional homology to diverse human proteins (see below).

EBNA-1 has been shown to block its own degradation (Levitskaya et al., 1997). Since viral proteins are normally broken down by proteasomes to peptides for presentation to cytotoxic T cells, the ability of EBNA-1 to inhibit its degradation allows the protein to avoid triggering the activation of cytotoxic T cells. EBV encodes proteins that inhibit apoptosis (see below). EBV LMP1 up-regulates the expression of several cellular proteins that inhibit apoptosis, including bcl-2 and A20 (Kulwichit et al., 1998). EBV infected Burkitt's lymphoma cells down-regulate the expression of several proteins that are important for killing by cytotoxic T cells. These include the transporter proteins associated with antigen processing that convey viral peptides from the cytoplasm to the endoplasmic reticulum for antigen presentation, the cellular adhesion molecules that allow the cells to contact with each other and the MHC class 1 molecules that allow cytotoxic T cells to recognize virus-infected cells (Khanna et al., 1999).

6.15 HUMAN PROTEIN HOMOLOGUES

BCRF1 AND IL-10

Interleukin-10 (IL-10) plays a critical role in EBV biology. EBV BCRF1 protein shows 84% sequence homology to human interleukin-10 (IL-10) (Vieira et al., 1991). BCRF1 protein is often termed as viral IL-10 (vIL-10). Human IL-10 has been described as a potent immunosuppressive sytokine: IL-10 inhibits activation and effector function of T cells, monocytes, and macrophages. Human IL-10 is otherwise a potent growth and activation factor for B cells and also protects some B cells from apoptosis (Moore et al., 1990 and 1991; Miyazaki et al., 1993; Thompson et al., 2004). The BCRF1 protein (viral IL-10) mimics the activity of interleukin-10 by inhibiting interferon-(gamma) synthesis by human peripheral blood mononuclear cells in vitro (Hsu et al., 1990). The BCRF1 protein (viral IL-10) is thought to play a role in the establishment of latent infection by suppression of the host immune system (Thompson et al., 2004). The BCRF1 protein (viral IL-10) is expressed late in the productive cycle and exhibits many properties ascribed to human IL-10 (de Waal Malefyt et al., 1991; Miyazaki et al., 1993; Swaminathan et al., 1993; Mahot et al., 2003). The viral transcription factor EB1, which is the main inducer of the EBV productive cycle, also activates

transcription of the human IL-10 gene and secretion of human IL-10 (Mahot et al., 2003). Therefore, an increase in IL-10 production occurs during latency and early and late during productive cycle (Mahot et al., 2003). This might favour the survival of EBV-infected cells.

BDLF2 AND CYCLIN B1

On the basis of amino acid sequence alignment, homology between the BDLF2 protein and human cyclin B1 has been suggested, although functional homology between these two proteins has yet to be confirmed (Hayes et al., 1999). Human cyclin B1 regulates the G2-M transition in the cell division cycle by activating particular cyclin-dependent protein kinases. Not so much is known about BDLF2 (Thompson et al., 2004). It has been detected in oral hairy leukoplakia but not in other diseases characterized by latent infections. It has been suggested to be a gene expressed late during the lytic cycle and BDLF2 may have a role in protecting the cell from immune elimination and programmed cell death (Hayes et al., 1999).

BHRF1, BALF1 AND BCL-2

Interestingly, EBV encodes two Bcl-2 homologues BHRF1 and BALF1. BHRF1, an early lytic cycle antigen, shows partial (25%) sequence homology to the human protein Bcl-2, a cellular anti-apoptotic protein (Henderson et al., 1993). In addition to structural homologue, BHRF1 is also fuctionally homologous to Bcl-2. BHRF1 may enhance cell survival, allowing oncogenic mutations to accumulate; it may also permit the production of a maximum number of virions through the inhibition of apoptosis (Oudejans et al., 1995; Thompson et al., 2004). Some EBV-positive lymphomas have been found to be positive for BHRF1 transcripts and latent BHRF1 transcripts have been detected in T cell lymphomas and in EBV-transformed tightly latent B-cell lines in vitro (Austin et al., 1988; Oudejans et al., 1995; Xu et al., 2001; Howell et al., 2005). EBV encodes also the other protein, BALF1, which has structural homology to Bcl-2 and is likely to have similar anti-apoptotic effects (Marshall et al., 1999; Bellows et al., 2002).

BARF-1 AND INTRACELLULAR ADHESION MOLECULE 1

The EBV BARF-1 protein shows some homology to the intracellular adhesion molecule 1, as well as to the human colony stimulating factor 1 receptor (Wei et al., 1994; Strockbine et al., 1998). BARF-1 is involved in immune suppression by either functioning as a soluble receptor for colony-stimulating factor 1 (which normally enhances the expression of interferon-α by monocytes) or by occupying intracellular adhesion molecule 1 receptors on T lymphocytes without leading to the proper stimuli necessary for T-cell activation (Wei et al., 1994; Sbih-Lammali et al., 1996; Cohen et

al., 1999; Thompson et al., 2004). Because interferon-γ and interferon-α inhibit the outgrowth of EBV-infected cells in vitro, the BCRF1 and BARF1 proteins may help the virus to evade the host's immune system during acute EBV infection or reactivation (Cohen, 2000).

6.16 CLINICAL SYNDROMES ASSOCIATED WITH EBV

6.17 INFECTIOUS MONONUCLEOSIS

EBV primary infection of young adults causes infectious mononucleosis. EBV is present in the saliva of most persistently infected individuals and is generally thought to be spread by close oral contact. However, there have been several reports of EBV in genital secretions, suggesting the possibility of sexual transmission between adults (Sixbey et al., 1986; Israele et al., 1991; Crawford et al., 2002). Recently, Thomas and colleagues (2006) investigated the risk of sexual transmission; low levels of virus detected in genital secretions compared to saliva suggest that this is not a major transmission route. The findings of small quantities of cell-associated virus suggest a latent infection, thus EBV is probably in the B lymphocyte rather than in the epithelial cell component of the secretions (Thomas et al., 2006).

The symptoms of infectious mononucleosis are thought to be caused by T cell activation and cytokine production (Foss et al., 1994; Andersson, 1996). Williams and colleagues (2004) suggested that T cells expressig CD244 and surface lymphocyte activation marker (SLAM) are responsible for the clinical features of mononucleosis but the control of activation is maintained by parallel increased expression of SLAM-associated protein (SAP). Over 50 percent of patients have the triad of fever, lymphadenopathy and pharyngitis. Spleno- and hepatomegaly, and palatal petechiae are each present in more than 10 percent. Less common complications are spleen rupture, hemolytic anemia, thrombocytopenia, aplastic anemia, myocarditis, hepatitis, Guillain-Barre syndrome, and CNS infection. Most infants and children are asymptomatic or have nonspecific symptoms (Henke et al., 1950; Straus et al., 1993).

Most patients with infectious mononucleosis have leukocytosis with an absolute increase in the number of peripheral mononuclear cells, heterophile antibodies, elevated serum aminotransferase levels, and atypical lymphocytes. These are predominantly T lymphoblasts of CD8+ subset but include some CD4+ T cells and also activated natural killer (NK)-like cells with the CD16 or CD56 markers (Rickinson et Kieff, 2001). Most of the symptoms of infectious mononucleosis are

attributed to the proliferation and activation of T cells in response to infection. Up to a few percent of the peripheral blood B cells may be infected with EBV in infectious mononucleosis. Activation of B cells results in production of polyclonal antibodies, heterophile antibodies and occasionally cold agglutinins, cryoglobulins, antinuclear antibodies, or rheumatoid factor (Cohen, 2000).

Serodiagnosis of EBV primary infection is based on measurement of antibody reactivity for various EBV antigens, including viral capsid antigen (VCA), the early antigens (EA), and members of the Epstein-Barr virus nuclear antigen (EBNA) family. Characteristic for EBV primary infection are positive VCA IgM and IgG results and lack of EBNA-1 antibodies (Henle et al., 1974). Avidity of VCA IgG or EA IgG separates primary and secondary infections (De Ory et al., 1993; Andersson et al., 1994; Vetter et al., 1994; Gray et al., 1995; Weissbrich, 1998; Korhonen et al., 1999). Serodiagnosis of EBV primary infection by the diagnostic laboratory of the Helsinki University Hospital campus (first University of Helsinki; now HUSLAB) is based on positive VCA IgM together with VCA (or corresponding) IgG of low avidity.

The treatment of infectious mononucleosis consists of good supportive care, including adequate hydration; nonsteroidal anti-inflammatory drugs or acetaminophen for fever and myalgias; and (possibly) gargling with a two percent lidocaine (Xylocaine®) solution to relieve pharyngeal discomfort (Ebell, 2004). A meta-analysis of five randomized controlled trials involving 339 patients found that patients who took acyclovir had less oropharyngeal shedding at the end of therapy, but this treatment did not provide significant clinical benefit (Torre et al., 1999). Based on clinical experience and case reports, corticosteroids are recommended in patients with significant pharyngeal edema that threatens respiratory function (Ebell, 2004).

The risk of splenic rupture is estimated at 0.1 percent, based on a retrospective series of 8116 patients (Farley et al., 1992). Because of this, the patients are recommended to avoid athletics for at least three or four weeks after disease (Ebell, 2004). Several studies have examined the long-term outcomes of infectious mononucleosis. Between nine and 22 percent of patients reported persistent fatigue or hypersomnia six months after infectious mononucleosis (Candy et al., 2002).

After mononucleosis individuals have a four-fold increased risk for Hodgkin's lymphoma (Munoz et al., 1978). Hjalgrim and colleagues (2000) studied the risk of Hodgkin's lymphoma after infectious mononucleosis by population-based cohorts of 38562 infectious mononucleosis patients in Denmark and Sweden and concluded the risk to be specific: The standardized incidence ratio for

Hodgkin's lymphoma remained elevated for up to two decades after mononucleosis, but decreases with time. The standardized incidence ratio for Hodgkin's lymphoma tended to increase with at the time of diagnosis of mononucleosis (Hjalgrim et al., 2000). Following infectious mononucleosis, the standardized incidence ratio for Hodgkin's lymphoma at ages 15-34 years was 3.49, which was statistically significantly higher than the standardized incidence ratio for any other age group (Hjalgrim et al., 2000).

6.18 CHRONIC ACTIVE EBV INFECTION

In 1948, Isaacs described a prolonged clinical course of infectious mononucleosis lasting from months to years (Isaacs, 1948). During the late 1970s and early to middle 1980s, several groups described a protracted illness usually preceded by infectious mononucleosis but with persistent fatigue, headaches, myalgia, lymphadenopathy, and intermitted or low-grade fever (Tobi et al., 1982; Hellmann et al., 1983; Dubois et al., 1984; Jones et al., 1985; Straus et al., 1985). Unexpectedly, unusual profiles of antibodies to EBV were common in this syndrome. Specifically, the titres of viral capsid antigen (VCA) and early antigen (EA) IgG antibodies were substantially higher in patients compared to controls. Because of these serological patterns and clinical symptoms, it was proposed that the syndrome arose from a chronic EBV infection.

Chronic active EBV infection is a rare disorder that has been defined by the presence of three features: severe illness of more than six month's duration that begins as a primary EBV infection or that is associated with abnormal EBV serology; histologic evidence of organ disease and demonstration of EBV antigens or EBV DNA in tissue (Straus, 1988). There are often extreme elevations of virus-specific antibody titers. Chronic active EBV infection is a heterogeneous EBV-related disorder, ranging from a mild/moderate form to a rapidly lethal disorder. The mild to moderate form is more common in Western countries, while the lethal form is more common in Japan (Jones et al., 1987; Ishira et al., 1995; Kimura et al., 2001). In 1978, Virelizier and colleagues described a case of severe type of chronic active EBV infection (Virelizier et al., 1978). The affected girl had a chronic disease chracterized by fever, lymphadenopathy, interstitial pneumonitis, thrombocytopenia and polyclonal hypergammaglobulinemia. She had extremely high antibody titres against VCA and EA. Subsequently, in 1984, Joncas and colleagues described a similar type of chronic active EBV infection (Joncas et al., 1984).

The lethal form of chronic active EBV infection is characterized by multiple organ failure, hemophagocytic syndrome and development of lymphomas. The following clinical features are common: symptoms persisting for at least six months and associated with (1) either intermittent or persistent fever, lymphadenophathy, and hepatosplenomegaly, (2) a tendency for pancytopenia and polyclonal gammopathy, and (3) no apparent manifestation of a serious underlying disease (Virelizier et al., 1978; Joncas et al., 1984; Schooley et al., 1986; Kawa-Ha et al., 1987; Miller et al., 1987; Jones et al., 1988; Kikuta et al., 1988; Ishira et al., 1989). Pneumonitis is another clinical manifestation reported in the literature (Virelizier et al., 1978; Schooley et al., 1986). Other symptoms, such as debilitating fatigue, sore throath, lymph node tenderness and pain, headache, myalgia and arthralgia, are also encountered. Allogeneic stem cell transplantation is considered the only potentially curative treatment for the lethal form of chronic active EBV infection (Teshima et al., 1996; Imashuku et al., 1997; Takami et al., 1998; Fujii et al., 2000; Okamura et al., 2000). Chronic active EBV infection has two distinct clinical classifications depending on whether T cells or NK cells are predominantly infected cell type in the peripheral blood (Kimura et al., 2001). T cell-type infections are characterised by high fever, anemia, hepatomegaly, lymphadenopathy and high titers of EBV-antibodies. In contrast, NK cell-type infections exhibit large granular lymphocytosis, hypersensitivity to mosquito bites and high IgE levels (Kimura et al., 2001 and 2003, Kimura, 2006).

EBV infects T or NK cells in many severe cases, and it is believed that these cells escape immune surveillance because of the lack of EBV-specific immunity (Kuzushima, 1996; Kimura et al., 1995; Tsuge et al., 2001). However, the exact pathogenesis is not known. In one study, in most of the patients with chronic active EBV infection, EBV-specific CD8+ T cells were not detected (Sugaya et al., 2004). Indeed, low frequencies of EBV-specific CD8+ T cells may be one of the immunological features of chronic active EBV infection (Sugaya et al., 2004).

6.19 X-LINKED LYMPHOPROLIFERATIVE DISEASE

X-linked lymphoproliferative disease is an inherited disease of males unable to restrict EBV infection (Purtilo et al., 1977). The mutated gene on the X chromosome has been identified as SAP (signaling lymphocyte activation molecule [SLAM]-associated protein), also known as SH2D1A or DSHP (Sayos et al., 1998). This gene encodes a protein located on the surface of T cells, which interacts with two other proteins: SLAM, present on T and B cells, and 2B4, present on T cells and natural killer cells (Sayos et al., 1998; Tangye et al., 1999). The absence of a functional SAP in

patients with X-linked lymphoproliferative disease is thought to impair the normal interaction of T and B cells, resulting in unregulated growth of EBV-infected B cells (Purtilo et al., 1977 and 1979).

X-linked lymphoproliferative disease is associated with a high morbidity; and the overall outcome is poor (Purtilo et al., 1979). If the risk patients are identified, regular immunoglobulin replacement therapy has been given to protect them from EBV or other viral infections (Gaspar et al., 2002). However, in a number of cases, this has not prevented the development of the disease (Okano et al, 1990). At present, the only curative treatment is allogeneic stem cell transplantation (Vowels et al., 1993; Pracher et al., 1994; Seemayer et al., 1995; Gross et al, 1996).

During the acute phase of X-linked lymphoproliferative disease, treatment is difficult as demonstrated by the high mortality rate. Treatment using antivirals (aciclovir / ganciclovir / foscarnet), high-dose immunoglobulin, immunosuppressive agents, and IFN-α have been disappointing (Okano et al, 1990 and 1991). With the identification of the XLP gene, gene therapy is a forthcoming option.

6.20 CANCERS ASSOCIATED WITH EBV

6.21 NASOPHARYNGEAL CARCINOMA

Nasopharyngeal carcinoma is prevalent in Southern China, in Northern Africa and among Alaskan Inuits. Nasopharyngeal carcinoma has three etiologic factors, including genetic susceptibility, chemical carcinogens, and EBV infection (Chan et al., 2004). In Southern China nasopharyngeal carcinoma is the third most common malignancy and the incidence rate varies between 15 and 50 per 100 000 (Ho, 1978; Zeng et al., 1982; Chan et al., 2002). Nasopharyngeal carcinoma is associated with the HLA A2-B46 haplotype in the Chinese population, with two alleles in linkage disequilibrium, whereas HLA A2 and A11 in non-Chinese patients are protective against nasopharyngeal carcinoma (Hildesheim et al., 2002). Nasopharyngeal carcinoma occurs sporadically in the United States and Western Europe. Nearly 100 percent of anaplastic or poorly differentiated nasopharyngeal carcinomas contain EBV genomes and express EBV proteins (Cohen, 2000). The EBV genome is present in the transformed cells but not in the lymphocytes of the tumor. Clonal EBV genomes are found in the early preinvasive dysplastic lesions or carcinoma in situ, indicating that EBV infection precedes the development of nasopharyngeal carcinoma (Pathmanathan et al., 1995).

Nasopharyngeal carcinomas are epithelial neoplasms. Three histopathological types are recognized in the World Health Organization (WHO) classification (Shanmugaratnam et al., 1993). Type I is squamous cell carcinoma with varying degrees of differentiation. Type II is non-keratinizing carcinoma and type III can be considered undifferentiated carcinoma of the nasopharyngeal type. The histological types may be of prognostic significance (Chan et al., 2002). The most common presenting symptom is cervical lymphadenopathy, followed by nasal, aural and neurological symptoms (Chan et al., 2002).

Patients with nasopharyngeal carcinoma often have elevated titers of IgA antibody to EBV structural proteins (Cohen, 2000). Measurement of EBV-specific IgA is useful in early screening for nasopharyngeal carcinoma in southern China (de The G et al., 1986). An increase in EBV-specific antibody titers after therapy is associated with a poor prognosis, whereas a declining or constant level is a favourable sign (Halprin et al., 1986). The quantity of EBV DNA detected in blood correlates with the stage and prognosis of the disease (Wei et al., 2005). Radiotherapy with concomitant chemotherapy has increased survival, and improved techniques (such as intensity-modulated radiotherapy), early detection of recurrence, and application of appropriate surgical salvage procedures have contributed to improved therapeutic results (Chan et al., 2002; Wei et al., 2005). Screening of high-risk individuals in endemic regions together with developments in gene therapy and immunotherapy might improve the outcome further (Wei et al., 2005).

6.22 BURKITT'S LYMPHOMA

In 1958, Dennis Burkitt described a disorder associated with jaw tumors in African children (Burkitt, 1958). In 1961, the neoplasm was identified as a form of malignant lymphoma, and became an entity called Burkitt's lymphoma (O'Conor, 1961). Burkitt's lymphoma is a high-grade malignant lymphoma of small, noncleaved B cells. In equatorial Africa, Burkitt's lymphoma is associated with *Plasmodium falciparum* malaria (Cohen et al., 2000). Over 90 percent of these endemic cases are associated with EBV (Lenoir et al., 1984). Infection with malaria is thought to diminish the T-cell control of proliferating EBV-infected B cells and enhance their proliferation. Not more than 25% of sporadic Burkitt's lymphomas are EBV DNA positive and express the EBV nuclear antigen-1 (EBNA1) and nuclear RNAs (EBER) (Gutierrez et al. 1992; Niedobitek et al., 1995; Lehtinen, 1987; Lehtinen et Lehtinen, 1998; Subar et al., 1998).

Burkitt's lymphoma cells contain a chromosomal translocation involving chromosomes 8 and 14, 22 or 2 (Taub et al., 1982; Dalla-Favera et al., 1982; Leder et al., 1983; Klein et Klein 1985; Manilov et al., 1986). These translocations result in positioning of the c-myc oncogene (chromosome 8) near the immunoglobulin heavy-chain (chromosome 14) or light-chain (chromosome 2 or 22) constant regions, leading to deregulation of the c-myc gene expression. The dysregulated expression of c-myc results in increased complex formation between the myc protein and a DNA-binding max protein which by transcriptional activation enables progression of the cell cycle and lymphoproliferation (Inghirami et al., 1990; Sanlund et al., 1996; Henriksson et Luscher, 1996). In addition to the EBV positivity of Burkitt's lymphoma, similar geographical gradient exists in the distribution of the chromosome 8 breakpoints: far upstream of c-myc in endemic Burkitt's lymphoma, immediate 5' of c-myc in Latin American, and within the transcriptional unit in sporadic Burkitt's lymphoma (Barriga et al., 1988; Gutierrez et al., 1992).

Tissues from patients with Burkitt's lymphoma in Africa usually contain EBV DNA and express only one EBV protein, EBNA-1. The form of latency characteristic of Burkitt's lymphoma is often referred to as latency I. As in nasopharyngeal carcinoma, clonal EBV genomes are found in Burkitt's lymphoma, indicating that it arises from a single EBV-infected cell (Cohen et al., 2000). Interestingly, anti-VCA and anti-EA(R) antibody titers in the serum of patients with endemic Burkitt's lymphoma are considerably higher than controls and anti-VCA antibody titers are raised even before the clinical onset of the tumor (Henle et al., 1969; de The G et al., 1978; Geser et al., 1982; Rickinson et Kieff, 2001).

Although current literature is limited by a lack of randomized trials, Burkitt's lymphoma appears to be curable in a high proportion of cases if treated with aggressive multiagent chemotherapy regimens (Bociek, 2005). The use of autologous stem cell transplantation appears to benefit the patients with chemotherapy-sensitive relapses. The role of allogeneic stem cell transplantation for this disease remains uncertain (Bociek, 2005).

6.23 HODGKIN'S LYMPHOMA

Hodgkin's lymphoma is an uncommon malignant tumor of the lymphatic system where the Reed–Sternberg giant cells that are characteristic for Hodgkin's lymphoma, replace the normal lymphoid structure. EBV DNA has been detected in about 40 to 60 percent of patients with Hodgkin's tumors in the United States. At the cellular level, EBV-encoded RNA (EBER), latent membrane protein

(LMP) and the Epstein-Barr nuclear antigen (EBNA) have been localized in the Reed–Sternberg (RS) cells of the diseased patients (Grasser et al., 1994; Armstrong et al., 1998; Sleckman et al., 1998). The viral genomes are monoclonal (Weiss et al., 1989; Jarret et al., 1991; Gulley et al., 1994). Elevated levels of EBV antibodies have been observed in patients with Hodgkin's lymphoma even before diagnosis (Hesse et al., 1977; Mueller et al., 1989). Conclusive evidence of the causal relationship of EBV infection and infectious mononucleosis in Hodgkin's lymphoma was obtained from longitudinal cohort and case-control studies (Rosdahl et al., 1974; Kvåle et al., 1979; Mueller et al., 1989; Lehtinen, 1989; Hjalgrim et al., 2000).

EBV positivity in lymphoma tissue is discerned in ~70% of mixed cellularity Hodgkin's lymphoma, >95% of lymphocyte-depleted Hodgkin's lymphoma, and in 10–40% of cases of nodular sclerosis; the lymphocyte-predominant Hodgkin's lymphoma subtype is almost always EBV negative (Chapman et al., 1998; Thompson et al., 2004). Geographic variations of EBV positivity have also been studied; EBV positivity in Hodgkin's lymphoma is found in 65% of cases in Japan, 67% of cases in Mexico, 94% of cases in Peru, 92% of cases in Kenya, 41% of cases in Italy, and ~50% of cases in the United States (Zarate-Osorno et al., 1995; Chang et al., 1996; Leoncini et al., 1996; Tomita et al., 1996; Thompson et al., 2004). Free circulating EBV DNA is often present in the plasma of patients with Hodgkin's lymphoma. Musacchio and colleagues recently reported (2006) EBV DNA present in the plasma of 91% of patients with EBV-associated Hodgkin's lymphoma, and in all patients with HIV-associated disease. EBV DNA prevalence was higher in patients with advanced disease, irrespective of HIV status.

Patients with Hodgkin's lymphoma have an excellent prognosis with modern chemotherapy even if the disease is far advanced at diagnosis (Josting et al., 2002; Schmitz et al., 2005). The impact of EBV on clinical outcome is still controversial. Kwon and colleagues (2006) reported both EBER and LMP1 positivities were associated with longer progression-free survival in patients younger than 25 years of age. However, reverse trends were noticed in older patients. Keegan and colleagues (2005) reported that EBV presence was associated with better survival in young patients (<15 years old). In adults aged 15 to 44 EBV did not affect the outcome. In older adults (45 to 96 years) EBV presence was associated with poorer survival for patients with nodular sclerosis (Keegan et al., 2005).

6.24 NON-HODGKIN'S LYMPHOMA IN IMMUNOCOMPETENT INDIVIDUALS

EBV can also infect lymphoid cells other than B-cells. Several types of non-B-cell, non-Hodgkin's lymphoma are associated with EBV: nasal T/natural killer (NK)-cell lymphoma and angioimmunoblastic lymphadenopathy are the most directly associated with EBV (Jones et al., 1988; Weiss et al., 1992; Thompson et al., 2004). Nasal T/NK-cell lymphoma is characterized by progressive necrotic lesions in the nasal cavity, nasopharynx and palate (Jaffe et al., 1996). It has been called angiocentric lymphoma, lethal midline granuloma, or polymorphic reticulosis (Harris et al., 1994). Nasal T/NK lymphoma cells have several unique genotypic and phenotypic features. These features include an absence of T-cell antigens, expression of natural killer cell marker CD 56, and absence of T-cell receptor gene rearrangement (Harabuchi et al., 1990; Weiss et al., 1992; Kwong et al., 1997; Baumforth et al., 1999; Thompson et al., 2004). EBV is strongly associated with these lymphomas, almost all cases are positive for EBV (Harabuchi et al., 1990; Ho et al., 1990; Weiss et al., 1992; Kanavaros et al., 1993; Nagata et al., 2001; Thompson et al., 2004). Nasal T/NK-cell lymphoma is a relative rare disease associated with quite a poor prognosis and it is more prevalent in Asia than in Europe and in the United States (Lipford et al., 1988; Frierson et al., 1989; Ratech et al., 1989; Ho et al., 1990; Nakamura et al., 1997; Cheung et al., 1998; Nagata et al., 2001).

Angioimmunoblastic lymphadenopathy is a T-cell lymphoma in which expanding B-cell clones are often present beside the T-cell clones. EBV infection is mainly seen in the B lymphocytes and B immunoblasts, although the virus also occurs in rare neoplastic and nonneoplastic T cells (Weiss et al., 1992; Thompson et al., 2004). The presence of EBV in only a subpopulation of cells suggests that EBV infection is secondary to malignancy or that the viral genome has been lost from the malignant cell (Thompson et al., 2004). EBV-positive B cells have also been observed growing in peripheral T-cell lymphomas (Ho et al., 1998); EBV might be activated in latently infected B cells by the neoplastic T cells, and/or the EBV-positive B cells might play a role in maintaining the malignant T-cell process (Hojo et al., 1995; Ho et al., 1999; Thompson et al., 2004).

6.25 POST-TRANSPLANT LYMPHOPROLIFERATIVE DISEASE

Post-transplant lymphoproliferative disease (PTLD) forms a wide spectrum of disorders complicating immunosuppressive conditions of various forms. An increased incidence of lymphomas in immunosuppressed transplant recipients was first recognized in 1969, and further studies demonstrated that 90% of PTLDs are Epstein-Barr virus positive (Penn et al., 1969; Paya et al., 1999). The development of PTLD is linked to a deficient EBV-specific cellular immune response. In solid organ transplant recipients, this deficiency is caused by imunosuppression administered to prevent graft rejection; in hematopoietic stem cell transplant recipients, it is caused by high-dose chemotherapy and/or radiation administered as part of the conditioning regimen, manipulation of the graft to deplete T cells or immunosuppression to prevent graft-versus-host disease (GVHD).

There is no universally accepted definition of PTLD (Loren et al., 2003). The term generally refers to a spectrum of B-cell hyperproliferative states, and includes benign conditions such as infectious monucleosis-like illness, polyclonal lymphoid hyperplasia, and monoclonal malignancies such as B-cell (and occasionally T cell) lymphomas (Nalesnik et al., 1996; Baumforth et al., 1999; Loren et al., 2003). Recommendations for a formal classification of PTLD were established by two international consensus groups and published in 1999 (Paya et al., 1999). According to these guidelines, the term PTLD may encompass the full range of EBV-related lymphoproliferative states, including benign processes (Loren et al., 2003). However, when not otherwise specified, PTLD should refer to the neoplastic end of the PTLD spectrum (Loren et al., 2003). According to the WHO classification, PTLD can be classified into: (1) early lesions, generally represented by EBV driven polyconal lymphoproliferations and (2) true monoclonal diseases, including polymorphic PTLD and monomorphic PTLD; the latter further distinguished into Burkitt lymphoma/Burkitt-like lymphoma, diffuse large B-cell lymphoma and Hodgkin's lymphoma (Jaffe et al., 2001; Capello et al., 2005). Neoplasia should be defined by two of the following three characteristics: (1) destruction of the underlying lymph node architecture; (2) monoclonality (regardless of morphology); (3) evidence of EBV infection in the neoplastic cells (Loren et al., 2003).

PTLD may present with a diverse spectrum of clinical symptoms and signs, underscoring the need for a high index of suspicion in making the diagnosis. Although the highest incidence occurs in the

first year after transplantation, cases have been reported as late as nine years after transplantation (Gottschalk et al., 2005). Symptoms and signs are similar to those seen during EBV primary infection and include fever, sweats, generalized malaise, enlarged tonsils, and cervical lymphadenopathy or sepsis like syndrome with rapidly progressive lymphoma (Weinstock et al., 2006). PTLD may involve virtually any organ system, including the central nervous system, bone marrow, intestine, kidneys, liver, spleen and lungs. Often, diffuse disease is diagnosed only at autopsy in patients thought to have fulminant sepsis or severe GVHD.

Most lymphoproliferations that arise after solid organ grafts are of host cell origin, whereas those that occur after bone marrow transplantation are often derived from donor cells (Baumforth et al., 1999). Following organ transplantation the incidence rates of this potentially fatal syndrome vary according to the type of graft and the immunosuppressive regimen (Paya et al., 1999; Ellis et al., 1999; Pen, 2000; Kew et al., 2000; Hopwood et Crawford, 2000). The duration of immunosuppression, the dosage, and the number of agents used influence both the risk of post-transplant lymphoproliferative disease and its clinical pattern (Baumforth et al., 1999).

In association with allogeneic stem-cell transplantation (SCT), PTLD has been considered rare (incidence $\leq$ 1 percent), albeit with risk factors associated with severe immunosuppression, higher rates may be encountered (Zutter et al., 1988; Witherspoon et al., 1989; Baumforth et al., 1999; Ifthikharuddin et al., 2000). Risk factors such as HLA disparity, graft T-cell depletion, severe graft-versus-host disease (GVHD), anti-thymocyte globulin (ATG) treatment or anti-CD3 monoclonal antibodies may increase the risk to as high as 15-25% (Lucas et al., 1998; Zutter et al., 1988; Curtis et al, 1999). Consistent with the essential role of T cells in controlling the proliferation of latent EBV-infected B-cells, each of these risk factors is associated with prolonged and severe T-cell immunodeficiency (Weinstock et al., 2006). In fact, methods of T-cell depletion that selectively target T cell or T- and NK-cell populations confer a significantly higher risk of PTLD than methods that deplete both T- and B cells (Curtis et al., 1999; Cohen et al., 2005; Weinstock et al., 2006). The importance of T-cell dysfunction is highlighted by the fact that the majority of PTLD cases occur within the first year post-transplant, when the recipient is severely immunocompromised to prevent GVHD or graft rejection (Gottschalk et al., 2005).

Early diagnosis of PTLD is demanding. Significant attention has been focused on assays for the early diagnosis of PTLD for two reasons. First, clinical presentation can be non-specific and easily confused with other infections or GVHD (O´Reilly et al., 1997; Gottschalk et al., 2005; Weinstock

et al., 2006). Second, the disease tends to be aggressive, and the prognosis without specific treatment, poor (Zutter et al., 1988; Benkerrou et al., 1998; Lucas et al., 1998). However, general reduction of immunosuppression, donor lymphocytes or EBV-specific cytotoxic T cells, and infusions of anti-CD 20 antibody have yielded promising results (Papadopoulos et al., 1994; Ifthikharuddin et al., 2000; Kuehnle et al., 2000; Verschuuren et al., 2002). It is reasonable to assume that early treatment improves survival. Quantitative PCR (qPCR) for EBV DNA is a new, highly encouraging approach (Kimura et al., 1999; Niesters et al., 2000; Ohga et al., 2001; Wagner et al., 2001). Also, the efficacy of PTLD treatment can be monitored with EBV qPCR (Rooney et al., 1998; Yang et al., 2000; Meerbach et al., 2001).

6.26 GASTRIC CARCINOMA

In immunocompetent individuals, EBV infection also might be associated with gastric cancers. EBV presence varies from >90% in lymphoepithelioma-like gastric carcinomas to between 5 and 25% in gastric adenocarcinomas (Niedobitek et al., 1992; Pittaluga et al., 1992; Oda et al., 1993; Wu et al., 2000; Thompson et al., 2004). Whether EBV plays a pathogenic role in either of these two tumors is unclear (Pittaluga et al., 1992; Oda et al., 1993; Thompson et al., 2004). It has been proposed that in lymphoepithelioma-like gastric carcinoma, EBV spreads from the nasopharynx to the stomach (Iezzoni et al., 1995; Wu et al., 2000; Thompson et al., 2004). With regard to gastric adenocarcinomas, EBV may enter the gastric epithelium without the use of a receptor; by binding of IgA antibody with EBV particles derived from B lymphocytes and the uptake of these particles by gastric epithelial cells (Fukuyama et al., 1994; Thompson et al., 2004) or via a receptor other than CD 21 (Yoshiyama et al., 1997).

EBV exhibits a novel latency pattern in gastric adenocarcinomas that includes the production of BARF-1, and the absence of LMP-1 (Strockbine et al., 1998; Kume et al., 1999; zur Hausen et al., 2000). Although any mechanism in gastric malignancies relating EBV to tumorigenesis remains highly speculative, there is a delay in apoptosis in EBV-positive gastric carcinomas and a decrease in cellular differentiation (Biose et al., 1993; Kume et al., 1999; Wu et al., 2000; Thompson et al., 2004).

6.27 EBV AND BREAST CANCER

The association between EBV and breast cancer is still quite controversial: EBV has been detected in subsets of breast cancer tumors (Labrecque et al., 1995; Luqmani et Shousha, 1995; Bonnet et al., 1999; Fina et al., 2001; Arbach et al., 2006), but negative results have also been obtained (Chu et al., 2001; Deshpande et al., 2002; Hermann et Niedobitek, 2003; Murray et al., 2003). Low viral loads have been detected in breast cancer biopsy specimens, but the infected cells have not been clearly identified. Arbach and his colleaques (2006) detected EBV DNA by quantitative PCR in whole tumors and microdissected tumor cells. Half of tumor specimens contained EBV DNA with low copy numbers. In this study the viral load was highly variable from tumor to tumor and EBV genomes were heteroneously distributed in morphologically identical tumors cells (there were some clusters of isolated tumor cells with relatively high genome numbers while other tumor cells from the same sample may be negative for EBV DNA). Arbach and colleagues (2006) detected EBNA-1 and BARF-1 transcripts in almost all of the EBV-positive tumors and LMP-1 RNA in three of the 15 cases studied.

Hennard and colleagues (2006) have reported that the 2B4 monoclonal antibody, which have been commonly used to demonstrate EBNA1 protein in tumor samples, cross-reacts with the MAGE4 protein. The MAGE4 protein is a cancer testis antigen which is expressed in a number of tumor types (Hennard et al., 2006). The authors recommend that this 2B4 monoclonal antibody should not be used to screen tissue samples for EBV (Murray et al., 2003; Huang et al., 2003). The PCR studies of microdissected tissues show that a small percentage of tumors carry EBV (Arbach et al., 2006; Murray, 2006). Interestingly, EBER expression has not been detected; it remains to be seen whether EBER-negative form of latency really exists (Murray, 2006). In conclusion, in those EBV positive cases, virus is present at low copy numbers and detectable only in a fraction of tumor cells (Arbach et al., 2006; Murray, 2006). It is possible that although EBV does not have an etiologic role in the genesis of breast cancer, the virus might contribute to tumor progression (Arbach et al., 2006, Murray, 2006).

6.28 EBV AND HIV

T cells from patients with AIDS suppress EBV-infected B cells less effectively than do cells from immunocompetent individuals (Birx et al., 1986). Patients with HIV have increased amounts of EBV in their oropharyngeal secretions (Jenson et al., 1999). A decline in EBV-specific cytotoxic T cells and an elevated and increasing EBV viral load preceded the development of EBV-associated non-Hodgkin's lymphomas in patients with HIV infection (Kersten et al., 1997; Cohen, 2000).

Oral hairy leukoplakia occurs in HIV infected patients as well as in some immunosuppressed transplant recipients. It presents with raised, white, corrugated lesions of the oral mucosa. It is a non-malignant hyperplastic lesion of epithelial cells. EBV DNA and herpesvirus particles are present in the upper, keratinized epithelial cells of the lesions (Cohen, 2000). Multiple EBV strains are often present in the same lesion. Oral hairy leukoplakia lesions show active viral replication and expression of lytic viral proteins (Greenspan et al., 1985; Triantos et al., 1997).

Lymphoid interstitial pneumonitis occurs primarily in children, but also in adults infected with HIV. It is characterized by diffuse interstitial pulmonary infiltrates and alveolar septa are infiltrated with lymphocytes, plasma cells, and immunoblasts (Cohen, 2000). EBV DNA and proteins have been detected in the pulmonary lesions (Andiman et al., 1985).

About 50 to 60 percent of non-Hodgkin's lymphoma tumors in HIV-infected patients contain EBV DNA or proteins (Hamilton-Dutoit et al., 1991; Shibata et al., 1993). Most of the tumors are classified as either immunoblastic lymphomas of Burkitt-type, while a smaller number are large-cell lymphomas (Cohen, 2000). Virtually all AIDS-related central nervous system lymphomas are derived from germinal center B cells and almost always contain EBV DNA (MacMahon et al., 1991; Cesarman et al., 1999). The prognosis of HIV related primary central nervous system lymphoma is very poor, with median survival time not exceeding 2 months. Brain biopsy was the method of choice for the definitive diagnosis, but it was and remains an invasive procedure with morbidity and mortality as well as considerable costs in terms of patients' management and quality of life. The strict association between AIDS-related central nervous system lymphoma and EBV led to the suggestion that EBV DNA in cerebrospinal spinal fluid (CSF) might serve as a diagnostic marker, reducing the time required for diagnosis and allowing a minimally invasive approach

(Antinori et al., 1997; Cingolani et al., 2005). The clinical usefulness of this methodology has been largely demonstrated through clinical practice.

6.29 DIAGNOSIS OF EBV INFECTIONS

Serological diagnosis of EBV primary infection is classically based on detection of heterophile antibodies and on measurement of antibody reactivity for various EBV antigens, including viral capsid antigen (VCA), the early antigens (EA), and members of the Epstein-Barr virus nuclear antigen (EBNA) family (Table II). Characteristic for EBV primary infection are positive VCA IgM and IgG results and lack of EBNA-1 antibodies (Henle et al., 1974; Linde, 1996). EA IgG becomes detectable after VCA IgG and disappears within a few months (Henle et al., 1971). During convalescence, VCA IgM antibodies usually disappear and the EBNA-1 test becomes positive, while VCA IgG persists for life. However, even in immunocompetent seropositive individuals EBNA-1 antibodies can sometimes remain negative (Lamy et al., 1982; Horwitz et al. 1985; Kampmann et al. 1993; Linde, 1996), and more often in immunocompromised patients or in chronic mononucleosis (Henle et al., 1981; Miller et al., 1987; Kampmann et al., 1993).

A sign of viral reactivation can be the reappearance of VCA IgM antibodies, but VCA IgM in some patients remains detectable long after EBV infection even with no known reason (Schmitz et al., 1972; Sumaya, 1977). To date, no single commonly accepted serological criterion exists for EBV reactivation. Many different parameters have been used: rise of EA IgG or EA IgA titer (Hornef et al., 1995), seroconversion of EA IgM (Hornef et al., 1995), decrease of EBNA IgG (Quesnel et al., 1992; Taneichi et al., 1993; Hornef et al., 1995), increase of VCA IgG (Rahman et al., 1991; Quesnel et al., 1992; Glaser et al., 1994) or simultaneous positivity to EA IgM and EBNA IgG (Obel et al., 1996). The ZEBRA protein (BamHI Z EBV replication activator) controls the switch of EBV from a latent to a productive cycle (Countryman et al., 1985 and 1987; Chevallier-Greco et al., 1986; Lieberman et al., 1986), and ZEBRA IgG has been proposed as a serological marker for EBV reactivation (Maréchal et al., 1993). On the other hand, while ZEBRA antibodies are rarely detectable in healthy EBV seropositives (2-4%), they do not uncommonly (75-87%) occur in patients with nasopharyngeal carcinoma, but also during infectious mononucleosis (85%) (Joab et al., 1991; Mathew et al., 1994). Furthermore, it seems that in the ZEBRA protein different B-cell epitopes are associated with different EBV-associated diseases (Tedeshi et al., 1995). In Finland Lehtinen and colleagues (1993) have reported that elevated EBV EA and EBNA antibody levels were associated with a statistically significant excess risk of malignant lymphoma/leukaemia. These

elevated antibody responses may be due either to destruction of neoplastic EBV positive B-cells and/or to activation of latent EBV infection early in the lymphomagenesis.

Measurement of IgG avidity has been shown to be a powerful tool for differentiation of primary and secondary infections of various pathogens (Hedman et al., 1988, 1991 and 1993; Blackburn et al., 1991). Also in EBV serodiagnosis, avidity of VCA IgG or EA IgG separates primary and secondary infections both in immunocompetent and immunocompromised individuals (De Ory et al., 1993; Andersson et al., 1994; Vetter et al., 1994; Gray 1995; Weissbrich, 1998; Korhonen et al., 1999).

Some difficulties have been encountered in detecting EBV at the tissue level, which is necessary for tumor diagnosis. The low copy number of viral genomes and the restricted pattern of viral antigen expression limit the reliability of many standard techniques including Southern blot hydridisation, antigen detection and in situ hybridisation (ISH) of mRNA or DNA targets (MacMahon et al., 1994). On the other hand, sensitive techniques such as PCR can detect EBV in normal tissues. Monoclonal antibodies to the EBV latent proteins EBNA-2 and LMP-1 have been used to demonstrate viral expression in lymphoproliferative disease, Hodgkin's disease and nasopharyngeal carcinoma (Young et al., 1989; Pallesan et al., 1991; Brousset et al., 1992). Due to variable expression of EBV latent proteins and technical difficulties with fixed tissues, the failure to detect these proteins does not necessarily indicate the absence of EBV (MacMahon et al., 1994).

However, targeting of the EBER transcripts with the in situ hybridisation method has proved to be a sensitive and specific technique (MacMahon et al., 1994). EBERs are excellent targets for in situ hybridisation, because they are abundantly expressed during EBV latency with an estimated copy number of ten million per cell (Arrand et al., 1982; Howe et al., 1986; MacMahon et al., 1994). With EBER in situ hybridisation EBV has been localised in Reed-Sternberg and Hodgkin's cells but occasionally also in small benign-appearing cells in tumor tissue in Hodgkin's lymphoma (Weiss et al., 1991; Herbst et al., 1992; Khan et al., 1992). With this technique EBV can be detected in EBV-associated lymphoid and epithelial tumors, in benign lymphoid tissue and in cultured cell lines (MacMahon et al., 1994). Oral hairy leukoplakia and some hepatocellular carcinomas are the exceptions; EBERs are not expressed (Gilligan et al., 1990; Niedobitek et al., 1991; Sugawara et al., 1999).

For definitive PTLD diagnosis, a biopsy is required for morphological analysis, detection of EBV antigens by immunohistochemistry and EBER in situ hybridisation; furthermore, the clonality of B-

cell growth may be determined by immunoglobulin light chain type or rearrangement of the corresponding genes (Hanto et al., 1983; Nalesnik et al., 1988; Randhawa et al., 1992; Knowles et al., 1995). However, due to the rapidity of PTLD progression especially after SCT, the diagnosis must be obtained quickly. New PCR-based methods show promise in permitting fast quantitation of viral DNA in fluidic or cellular samples (Kimura et al., 1999; Limaya et al., 1999; Niesters et al., 2000; Hukkanen 2000 and 2002). Also the efficacy of PTLD treatment may be monitored by quantitation of EBV load, aiming at a favourable balance between the degree of immunosuppression and the preponderance of (graft or host) rejection (Rooney et al., 1998; McGuirk et al., 1999; Yang et al., 2000; Meerbach et al., 2001; Weinstock et al., 2006).

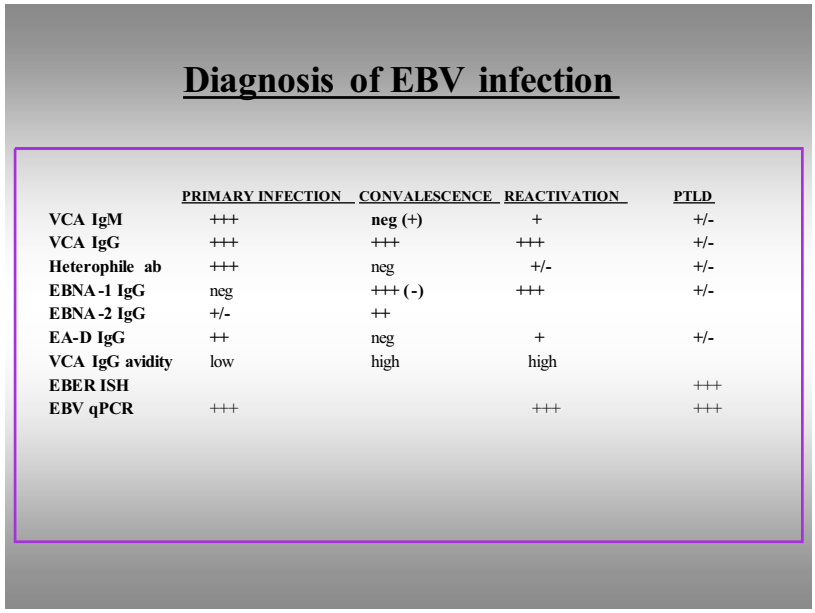

Table II. Summary of diagnostic EBV antibody, ISH and PCR findings.

7. AIMS OF THE PRESENT STUDY

(i) Our first goal was to assess the relation between the occurrence of VCA IgM together with high-avidity VCA IgG antibodies, and the presence of cytomegalovirus infection.

(ii) To set up an in situ hybridisation assay for detection of EBV RNA in tissue sections and for diagnosis of EBV infection at the cellular level.

(iii) To set up up a real-time PCR assay for quantification of EBV-DNA in serum or plasma samples for the diagnosis of PTLD.

(iv) To estimate the contributory role of EBV, cytomegalovirus and human papilloma viruses in the development of malignancies after heart transplantation.

(v) To determine the incidence of fatal Epstein-Barr virus –related PTLD in a large number of stem-cell transplantations, and to assess the diagnostic value of the real-time quantitative PCR for EBV-DNA.

(vi) To identify the risk factors for PTLD.

(vii) To evaluate the frequency of EBV reactivations and the kinetics of development of PTLD in a large cohort of unselected stem-cell transplant recipients.

(viii) To evaluate the development of PTLD according to the copy number levels of EBV DNA.

(ix) To find out the frequency and clinical significance of EBV infections of various types occurring after liver transplantation in adult recipients.

8. MATERIALS AND METHODS

8.1 PATIENTS (I, II, III, IV, V, VI)

Study I: Altogether, 192 samples of serum from 60 patients with serologically confirmed CMV primary infection, defined by seroconversion or ≥ fourfold rise of CMV IgG or low avidity of CMV IgG, together with detectable CMV-IgM antibodies, were assayed for EBV VCA IgM and IgG. Samples with diagnostic findings, seroconversion of VCA-IgM or ≥ fourfold rise in VCA-IgG, were further examined for VCA IgG avidity, EA IgG, EBNA IgG, HHV-6 IgG and as an immunofluorescence (IF) specificity control for nephropathia epidemica IgG (see below). For additional control, samples were studied for IgG antibodies against another latently infecting herpesvirus; varicella zoster virus.

According to clinical data, 50 patients (age range 3-64 years, mean 31) with 154 sera were immunocompetent. The cardinal symptoms were: fever, lymphocytosis, fatigue, and headache. The mean period between the diagnostic samples was 37 days. Of the 60 patients, ten (38 sera) were organ transplant recipients (2 heart, 3 liver, and 5 kidney) with posttransplant CMV primary infection (age range 17-61 years, mean 43). The serodiagnosis of CMV primary infection in the latter group was made 30 to 237 days (mean 105) after transplantation as indicated by the collection date of the first seropositive sample of each patient containing low-avidity CMV IgG. The mean period between the diagnostic samples in this group was 90 days.

Conversely, serum pairs from 22 patients (age range 1-42 years, mean 16) with EBV primary infection, diagnosed by positive EBV VCA IgM together with low-avidity VCA IgG, were studied for CMV IgM, IgG, and IgG avidity. The mean sample interval was 17 days. All the serum samples described had been referred for diagnostic purposes to the clinical virus laboratory of the Helsinki University Central Hospital.

As a separate control group, sera from 170 children (age range <1-15 years, mean 3.8) with acute septic or severe respiratory infection treated at The Hospital for Children and Adolescents, University of Helsinki, were studied for VCA IgM, and follow-up samples of the IgM positive sera were further studied for VCA IgG and IgG avidity. Serum pairs from these controls were also studied for HHV-6 IgG.

Study II: In total, 249 heart transplantations were performed in the Helsinki University Central Hospital between February 1985 and October 1999 (Mattila et al., 1997). The presence of EBV and human papilloma virus (HPV) and cytomegalovirus (CMV) was studied in 20 patients who developed malignancies after heart transplantation. The tumors were analyzed for the presence of HPV by PCR and for EBV by in situ hybridisation. CMV infection was verified by immunochemical quantitation of CMV antigen in peripheral blood (see below).

In October 1999, 170 (68%) of the patients were alive. The indications for heart transplantation were dilating cardiomyopathy (CMP) in 130 patients, ischemic heart disease (IHD) in 94, valve disease in 15, congenital heart disease in six, and myocarditis in four cases. The immunosuppression consisted of anti-thymocyte globulin (ATG), cyclosporine A (CyA), azathioprine (Aza) and methylprednisolone (MP). ATG (Fresenius or Merieux) at 2.5mg/kg was given during the first 3 post-operative days. CyA was started on the first post-operative day aiming at level 250-350 μg/L during the first 3 post-operative months and then tempering down to 150-200 μg/L during the first post-operative year. Aza at 2 mg/kg was started on the first post-operative day. The dosage was reduced if the white blood cell count per mL dropped to less than 3000. One gram of methylprednisolone was given during the operation and subsequently for 3 days. The dose was then tapered down to 0.1 mg/kg for the first month post-operatively. Acute rejection was diagnosed by endomyocardial biopsy and treated with 0.5-1.0 g of methylprednisolone for 3 days, after which an endomyocardial biopsy was taken. If the rejection had not subsided, ATG was administered. Acyclovir 800mg daily was given for 6 wk to all patients for prophylaxis against infections of the herpes virus group, and trimethoprim-sulfa-methoxazole was given three times a week for 6 months for prophylaxis of *Pneumocystis jirovecii* infections.

Study III and IV: During 1994-1999 257 adult patients underwent allogeneic SCT at Helsinki University Central Hospital, 132 (51%) recipients were alive and 125 (49%) had succumbed by June 2001. The cause of death was transplant-related in 76 patients (61%), relapse in 42 patients (34%), and other in seven cases (5%). Seventy-seven (62%) of the 124 deceased patients underwent autopsy. Their post-morten reports were reviewed in purpose of finding possible PTLD cases. In suspect cases archived post-mortem paraffin blocks were re-examined immunohistologically for EBV antigens (LMP, EBNA) and by in situ hybridisation for EBV RNA (EBER 1 and 2) (see below). From a subset of the PTLD cases (N=12) and a series of corresponding stem-cell recipient

controls (N=36), consecutive samples of serum (N=103 and 364, respectively) were studied by qPCR for EBV DNA, and the clinical data were reviewed.

Of the donors 173 were siblings and 84 were unrelated. Most (250) transplants were carried out with a conventional myeloablative regimen consisting of cyclophosphamide and total body irradiation (TBI), and in transplants from an unrelated donor also antithymocyte globulin (ATG) was also used. In seven transplantations, the conditioning was non-myeloablative; fludarbine combined with cyclophosphamide or a single 2 Gy dose of TBI. The indication for transplantation was a hematological malignancy in 253 patients and aplastic anemia in four patients. All donors, except one sibling with a single HLA A locus difference, were matched for HLA A, B, and DR. The grafts were non-manipulated apart form red cell and plasma removal from bone marrow grafts in cases of ABO-incompatibility. For graft versus host disease (GVHD) prophylaxis in myeloablative transplantation, the patients received cyclosporine A (CyA), short-course methotrexate, and methylprednisolone (MP) (Ruutu et al., 2000); and in non-myeloablative regimens, CyA with mycophenolate. Acute GVHD was assessed and graded according to previously published criteria (Thomas et al., 1975) and treated with intravenous MP. In corticosteroid-resistant cases ATG was given as second-line treatment.

As a positive-control group for the EBV qPCR assay (see below), sera were collected from 16 patients with serologically verified EBV mononucleosis (detectable EBV-IgM together with low avidity of EBV-IgG) 4-22 days (mean 11) after clinical onset. Of these mononucleosis patients 94% (15/16) presented with EBV DNA in serum, with copy numbers ranging from 4400 to 249 300 (mean 45 700) per ml. As negative-control groups, we had 13 members of healthy laboratory staff and eight patients with non-EBV tonsillitis. No sera from these controls contained detectable levels of EBV DNA.

Study V: In total, 105 adult liver allograft recipients transplanted between 1999-2002 were sequentially monitored for CMV, HHV-6 and HHV-7 during the first year after transplantation, and the specimens were retrospectively used to investigate the presence of EBV by qPCR (see below). As basic immunosuppressive therapy, the patients received combinations of steroids, azathioprine, and cyclosporine or tacrolimus. Rejections were treated with high doses of steroids. No routine antiviral prophylaxis was given, besides ganciclovir (5mg/kg twice daily) during rejection therapy. CMV infections were diagnosed using the standard pp65-antigenemia assay and by quantitative plasma DNA-PCR (Cobas Amplicor CMV Monitor, Roche, or real-time TaqMan PCR).

Symptomatic CMV infections were treated with intravenous ganciclovir (5mg/kg twice daily) for at least two weeks. As all our transplant recipients were adults, pre-transplant EBV serology (available for 61/105) showed a seroprevalence of 97%; post-transplant EBV serology was performed only by demand.

The clinical material comprised 1284 consecutive samples of EDTA-blood obtained weekly during the patients´ hospitalization and thereafter according to our clinical protocol at 1, 2, 3, 6, 9 and 12 months after transplantation and in case of clinical symptoms. The blood sampling for the EBV DNA-PCR occurred in parallel with the sampling for the CMV-pp65 antigen test, CMV DNA-PCR and HHV-6 and HHV-7 antigen detection, which were performed immediately. Plasma was isolated within 24 hours of EDTA-blood and used for PCR. Plasma samples were stored at -70°C for later retrospective analysis.

Study VI: Altogether 409 adult patients were treated with haematopoietic stem cell transplantation for hematological malignancy or severe aplastic anemia during the years 1988 – 1999 at Helsinki University Central Hospital (HUCH). Four hundred and six patients were included in the study. Altogether 5479 serum samples from 406 adult allogeneic SCT recipients treated during 1988-1999 at Helsinki University Central Hospital were studied by qPCR for EBV DNA. The patients in the studies III and IV are also included in this patient material. Three patients were excluded due to missing serum samples. The sera had been collected sequentially, 2 to 28 samples from each patient. The clinical and transplant data of the patients are described in detail in Juvonen et al. (2007).

8.2 SEROLOGICAL DIAGNOSIS OF EBV INFECTION (I, III)

EBV VCA IgM was determined by an immunofluorescence assay (IFA) (Gull Laboratories, Salt Lake City, UT), in which rheumatoid factor interference was prevented by GullSORB IgG Inactivation Reagent (Gull Laboratories). VCA IgG was measured by an EBV IgG enzyme immunoassay (EIA) (Gull Laboratories) performed according to the manufacturer's instructions. EA IgG was measured by IFA (Gull Laboratories). This test detects both diffuse (D) and restricted (R) components of the EBV EA complex. EBNA IgG was measured with anticomplement IFA (Gull Laboratories) using as antigen Raji cells, which preferentially express EBNA-1. Heterophile antibodies were determined by the agglutination slide test Monosticon DRI-DOT (Organon

Teknika, Boxtel, Netherlands). VCA IgG avidity was measured on EBV VCA EIA plates (Gull Laboratories) by a protein-denaturing EIA employing endpoint titration of IgG (Hedman et al., 1989 and 1993). Briefly, the sera were diluted in PBS containing 0.05% Tween 20 (PBST) serially in fourfold steps from 1:40 to 1:10,240, or from 1:10 to 1:2560, depending on the VCA IgG titer. After 1 hr incubation at 37°C, the wells containing the first four (lower) dilutions were washed with 6 M urea in PBST and those with the last four (higher) dilutions with PBST alone. All wells were then treated for 30 min at 37°C with alkaline phosphatase-conjugated antihuman IgG followed by substrate. Two antibody titration curves were constructed for each sample: one of the urea-washed wells and the other of the PBST-washed wells. IgG avidity was calculated by the percent ratio of antibody titers (urea+/urea-) x 100. Avidity ≤25% indicates primary infection; 25-40% is borderline; and ≥40% indicates past immunity (Hedman et al., 1993; data not shown). In three cases with controversial results, EBV-IgG avidity was measured by the Enzygnost EBV IgG kit (Behring Diagnostics, Marzburg, Germany) containing an EBV antigen mix employing single dilutions of serum (Hedman et al., 1988) as described by Weissbrich (1998).

8.3 SEROLOGICAL DIAGNOSIS OF CMV, HHV-6, NEPHROPATHIA EPIDEMICA AND VARICELLA ZOSTER (I)

CMV IgM and CMV IgG were measured by indirect EIA (Labsystems, Helsinki, Finland) according to the manufacturer's instructions. CMV IgG avidity was measured with EBV IgG-EIA kits (Labsystems) essentially as described above for VCA IgG avidity. CMV-IgG avidity ≤20% indicates primary infection; 20-35% is borderline; and ≥35% denotes past immunity (Hedman et Andersen, 1993). HHV-6 IgG was measured by an in-house IFA using infected HSB-2 cells as antigen as described previously (Linnavuori et al., 1992). Nephropathia epidemica IgG was determined by IFA with Puumala virus-infected Vero E6 cells (Hedman et al., 1991). The pattern of IgG fluorescence indicating acute infection or past immunity was evaluated and categorized as shown before (Vapalahti et al., 1995). Varicella zoster IgG was measured by indirect EIA on polystyrene strips (Labsystems) coated with varicella zoster EIA antigen (Virion, Rüschlikon, Switzerland).

8.4 DIAGNOSIS OF CMV (II, V)

CMV infections were diagnosed by the presence of pp65 antigen-positive leukocytes (100 positive cells per 50 000) in peripheral blood as determined by immunochemical staining with monoclonal

antibody (Biotest Pharma, Frankfurt, Germany) (Koskinen et al., 1993) in patients with a clinical picture compatible with CMV disease (fever, leucopenia, thrombocytopenia, hepatopathy, arthralgia, gastroenteritis, or pneumonia). Before 1988, when the CMV antigen test was not available, CMV infections were diagnosed by the appearance of inclusion bodies in cytology samples obtained by bronchoalveolar lavage, by the appearance of CMV-specific IgM antibodies, or by a fourfold rise in CMV-specific IgG antibodies as detected by the respective indirect enzyme-linked immunoassays (Labsystems, Helsinki, Finland).

In study V for detection of CMV the standard CMVpp65 antigenemia test (see above) and quantitative CMV-DNA PCR were used. Quantitative plasma CMV DNA-PCR (Cobas Amplicor CMV Monitor, Roche, or real-time TaqMan PCR) was used for quantitation of CMV DNA in plasma (Piiparinen et al., 2002 and 2004).

8.5 DIAGNOSIS OF HPV (II)

In the 15 patients with epithelial malignancies eight tumors occurring in seven patients were studied for HPV DNA. Five skin cancers (two samples from the same patient), one carcinoma of the tonsil, one of the tongue, and one of the lip were studied. HPV DNA was detected by PCR. Two sets of primers CPIV/CPV and GP5+/GP6+, were used for each tumor. The CPIV/CPV primers detect a variety of mucosal and cutaneous HPV types (Tieben et al., 1994) and the GP5+/GP6+ primers a large number of mucosal HPV types (De Roda Husman et al., 1995). The PCR conditions for the CPIV/CPV were 2 mmol/l $MgCl_2$, 0.2 mmol/L of each dNTP, 0.4 μmol/L of each primer, 1 U AmpliTaq Gold (Perkin-Elmer, Foster City, CA, USA) in 50 μL, and the PCR run consisted of 10 min of hot start at 95°C and 35 three-step cycles of 30 s at 95°C, 60 s at 50°C, 60 s at 72°C. The PCR conditions for the GP5+/GP6+ were 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 0.4 μmol/L of each primer, 1 U AmpliTaq Gold in 50 μL. The PCR consisted of 10 min of hot start at 95°C and 40 three-step cycles of 60 s at 95°C, 120 s at 40°C, and 90 s at 72°C. The PCR run ended with 4 min at 72°C. One-fifth of the PCR product was run in an agarose gel, stained with ethidium bromide, and visualized under UV light. The amplified fragment from one of the HPV DNA-positive patient was cloned and sequenced to determine the HPV type.

8.6 DIAGNOSIS OF HHV-6 AND HHV-7 (V)

HHV-6 infections were identified by the qualitative HHV-6 antigenemia test (Lautenschlager et al., 2000), which detects the virus-specific antigens in peripheral blood mononuclear cells (PBMCs). The presence of viral antigens in the cytopreparations was demonstrated by indirect immunoperoxidase staining with monoclonal antibodies (MAB8533 and MAB 8535; Chemicon Inc.,Temecula, CA, USA) against an early HHV-6 specific antigen (variant A and B), and a HHV-6 variant B virion protein, as described previously (Lautenschlager et al., 2000). HHV-7 specific antigens were detected in the cytocentrifuge PBMC preparations by using immunoperoxidase staining with two monoclonal antibodies (Biodesign International, Saco, ME, USA) to detect the early and late (gp110 and gp160) antigens of HHV-7, respectively, as described previously (Lautenschlager et al., 2002).

8.7 DIAGNOSIS OF MALIGNANCY (II)

Diagnosis of malignancy was based on microscopy of biopsy specimens. Lymphoma diagnosis was based on immunohistochemical staining of the tissue specimens with antibodies against CD45, CD19, and CD3 antigens. Lymphomas were classified according to the Revised European-American Lymphoma classification (Harris et al., 1994), because this lymphoma classification was the mostly used in those days. Two of the lymphomas were Burkitt's (patient 17 and 19), one located in the submandibular lymph nodes (patient 19) and the other in the ileum (patient 17). Three patients had CD20 + diffuse large cell lymphomas (patients 15, 16, and 18), two were located in lymph nodes of the neck (patients 15 and 18), and one in retroperitoneal lymph nodes (patient 16). In one case, involvement of the ileum was also seen in addition to lymph nodes of the neck (patient 15). Patient 20 had peripheral T-cell lymphoma.

8.8 B-CELL CLONALITY (III, IV)

Clonality of B-cells was determined by genomic DNA extracted from paraffin sections (Jee et al., 2001) by PCR for immunoglobulin gene rearrangement (Welterlin et al., 2000). The primers were: VH1, 5′-CTG TCG ACA CGG CCG TGT ATT ACT G-3′; 5′-GGG TCC CTG AGA CTC TCC TGT GCA-3′; VH1/VH3, 5′-AAC TGC AGA GGA GAC GGT GAC C-3′; CT (3A), 5′-TCA GAG TGG GCA CAT GTT GG-3′; CT (3B), 5′-TTC CTA CCC TGC CAT CCA TC-3′. The detection threshold of this method is 1-5% clonality. With paraffin-embedded tissues the method is highly specific but only moderately (~80%) sensitive (Welterlin et al., 2000).

8.9 EBV IN SITU HYBRIDISATION (II, III, IV, V, VI)

To detect EBV in the malignant cells, the tumor tissues were studied by in situ hybridisation for Epstein-Barr-encoded RNA (EBER 1 and EBER 2) (MacMahon et al., 1991; McClain et al., 1995). Fixed paraffin sections of the malignant tissues were permeabilized proteolytically by proteinase K. In situ hybridization was performed using a fluorescence-labeled peptide nucleic acid probe complementary to EBER 1 and EBER 2 (Dako, Glostrup, Denmark) and assayed by Ventana ES automated slide stainer (Ventana Medical Systems, Tucson, AZ, USA). The probe was visualized by anti-fluorescein antibody (Boehringer Mannheim, Mannheim, Germany) and Ventana DAB detection kit (Ventana Medical Systems). Hematoxylin was used as counterstain. To confirm preservation of RNA, separate slides of each sample were assayed with a positive control PNA probe (Dako) directed against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA. A random PNA probe (Dako) was used as a specificity control.

8.10 EBV qPCR (III, IV, V, VI)

For the qPCR assay, DNA was purified from 200 µl serum by proteinase K digestion (0.5mg/ml in 10 mM Tris-buffer, pH 8.0, containing 10 mM NaCl 1mM EDTA and 0.5% SDS) overnight at 37 °C, followed by phenol and chloroform extraction and ethanol precipitation. The purified DNA was diluted in 50 µL, of which 5 µL was used as a template for the qPCR reaction (Heid et al., 1996). The primers amplifying a conserved sequence of viral DNA polymerase (BALF5) gene and a fluorogenic probe for this area have been described by Kimura and colleagues (1999). The probe was synthesized by PE Applied Biosystems (Foster City, CA, USA). Five microliters of purified DNA was added to a PCR mixture containing 1 x TaqMan Universal PCR Master Mix (PE Applied Biosystems), 0.3 µmol of each primer and 0.2 µmol of the fluorogenic probe. Following 2 min at 50 °C for AmpErase UNG enzyme activity and 10 min at 95 °C for activating AmpliTaq Gold DNA polymerase, 40 cycles of 15 s at 95 °C and 1 min at 60 °C were carried out by a model 7700 Sequence Detector (PE Applied Biosystems).

Real-time fluorescence was measured, and the threshold cycle value (Ct) for each sample was calculated (Heid et al., 1996). For each PCR run, a standard curve was prepared by sequential logarithmic dilutions of EBV DNA extracted from 6.00×10^9 EBV particles, strain B95-8 (Advanced Biotechnologies Incorporated, Columbia, MD, USA). The dilutions corresponded to

10^6-10^1 EBV particles per reaction. The curve was created with ABI 7700 Sequence Detection System software by plotting the Ct values against the known EBV DNA concentration. The detection limit for DNA positivity was 500 copies per ml serum. All the samples were tested in duplicate of which the mean value was taken as the DNA copy number. If the difference of the duplicate Ct values was more than 0.5 cycles, the PCR reaction was performed again. Besides a standard curve obtained with EBV control DNA, each run included several negative and non-template controls, and a positive control containing EBV-DNA of a known copy number.

Diagnostic sensitivity was calculated among the 12 cases by the number of qPCR-positive samples relative to the number of samples drawn after clinical onset of PTLD. Diagnostic specificity was calculated among the 36 SCT recipient controls by the number of qPCR-negative samples relative to the total number of samples.

Some modifications were made in study V: Plasma of the specimens, which were obtained for routine monitoring of the betaherpesviruses and stored for later analysis, were retrospectively investigated for EBV-DNA. EBV DNA was isolated by using the MagNa Pure LC Instrument (Roche). MagNa Pure LC Total nucleic acid isolation kit (Roche) was used for extraction of total nucleic acid from 200µl of plasma samples. The purification followed the manufacturer´s instructions. The nucleic acid was eluted in 50µl of low salt elution buffer, where of 12.5 µl was used for each PCR run.

In study VI the sera were assayed retrospectively in pools of five, each comprising sequential sera from the same patient when possible. If the pool was EBV qPCR positive with a detection limit for DNA positivity of 500 EBV copies/ml, its constituent sera were re-examined individually. Samples with positive results were re-examined. Before onset of this study, we carefully set up the pooled-sample PCR approach, by investigating a large number of serum samples with a known, variably high EBV DNA level (103 sera from 12 PTLD cases presented in III; data not shown).

8.11 STATISTICAL ANALYSIS (IV)

Study IV: The χ^2 –test with continuity correction or Fisher's exact test, when appropriate, was used to compare (graft-versus-host disease) GVHD and the use of (anti-thymocyte globulin) ATG in relation to the risk of PTLD. The role of GVHD was examined by cross-tabulating PTLD and ATG, also separately, in the groups with or without GVHD.

9. RESULTS

9.1 CMV-INDUCED EBV IMMUNOREACTIVATION (I)

In serological diagnosis of mononucleosis we had not uncommonly discovered high-avidity VCA IgG antibodies, indicating past immunity, in EBV VCA IgM positive patients. Further studies often revealed CMV primary infection. Here, we wanted to determine systematically if and how frequently CMV induces such findings.

We assayed 192 samples of serum obtained from 60 patients with serologically confirmed CMV primary infection. Among the 50 immunocompetent patients with CMV primary infection, 46 had past immunity for EBV. Altogether 18 (38%) patients had VCA-IgM antibodies in one or more sera. In analysis of sequential samples, eight patients showed antibody profiles of EBV reactivation: seroconversion of VCA IgM and/or ≥ fourfold rise of VCA IgG, together with high or intermediate avidity of VCA IgG. All eight patients were EBNA IgG-seropositive; one of these showed a fourfold titer rise in EBNA IgG. EA IgG was detectable in most of the patients. Of these patients with EBV reactivation, half had in their first samples high (>40%) VCA IgG avidity and the other half had borderline (25-40%) avidity. In one patient, the VCA IgG avidity decreased during follow-up, finally reaching a low level. However, with the EBV antigen mix, IgG avidity of this patient remained constantly high, confirming that this patient had past immunity for EBV. Among these eight patients only one was barely and transiently positive for heterophile antibodies and another had a borderline result; the remaining six patients had no detectable heterophile antibodies.

Interestingly, all eight patients with both a CMV and an EBV diagnosis had also high HHV-6 IgG titers; four had ≥ fourfold titer rises in HHV-6 IgG. Of these eight patients with EBV antibody rises, two had Puumala-virus IgG antibodies of past immunity; non-specific antibodies against uninfected Vero E6 cells were not observed.

The clinical data of the patients with CMV primary infection-induced EBV antibody rises were compared with those having CMV primary infection without signs of EBV infection. In all, the clinical pictures in these two groups were similar. However, the patients with CMV-induced EBV

"immunoreactivation" reported a sore throat somewhat more frequently (P=0.04) than did those with CMV primary infection alone.

Of the 60 patients with CMV primary infection, 10 were organ transplant recipients. All of them were EBV-seropositive; five showed serological pictures of EBV recurrence. Of these five patients, three had ≥ fourfold increases of VCA IgG and three had seroconversions of VCA IgM. Five patients had EBNA IgG; none showed decreasing titers, whereas one had a fourfold titer rise in EBNA IgG. EA IgG was detectable in 2/5 patients, of which four had in their first samples high (<40%) VCA IgG avidity and one had borderline (25-40%) avidity. Two patients showed decreases of VCA IgG avidity, whereas with the EBV antigen mix such avidity decreases were not seen. None of these five patients had heterophile antibodies. Concomitant with their serological pictures of EBV reactivation, all five patients demonstrated ≥ fourfold titer rises in HHV-6 IgG. One patient had past immunity for Puumala virus, while non-specific Vero-cell antibodies were not seen. Of the total of 60 patients with CMV primary infection, 58 were varicella zoster virus seropositive. The varicella zoster IgG antibodies rose significantly in only one patient.

Conversely, 22 patients with EBV primary infection were studied for CMV IgM, IgG and IgG avidity. Among these patients, 10 were CMV-seropositive, and none presented with a CMV serodiagnosis. As a separate control group, sera from 170 children with acute septic or severe respiratory infection were studied for EBV and HHV-6 serology. Among the 170 controls, only two were EBV IgM-seropositive; both had low avidity of VCA IgG, indicating EBV primary infection, whereas none showed a picture of EBV immunoreactivation. Similarly, only 3/170 (2%) controls showed diagnostic rises in HHV-6 IgG.

We concluded that CMV is a particularly active immunoactivator of some, but not all, members of the herpes virus family and suggest that the in vivo interplay between CMV and EBV occurs unidirectionally. The high frequency of heterologous herpes virus immunoreactivations poses demands on – but also provides useful opportunities for laboratory diagnosis of herpesvirus infections.

9.2 MALIGNANCIES AFTER HEART TRANSPLANTATION (II)

Altogether, 249 heart transplantations were performed in the Helsinki University Central Hospital during 1985-1999. Of these transplant recipients, 20 (8.0%) developed malignancies during the follow-up period (mean 6.5 yr). Fourteen had epithelial malignancies, five had lymphoma, and one had both lymphoma and spinocellular carcinoma of skin. Among epithelial malignancies were four pulmonary tumors, two urinary bladder carcinomas, one renal adenocarcinoma, one epidermoid carcinoma of the tongue, one spinocellular carcinoma of the lip, one melanoma of the skin, two basal cell carcinomas of the skin, five case of Morbus Bowen of the skin, and four spinocellular carcinomas of the skin. We determined the presence of EBV, human papilloma viruses (HPV) in these tumors and quantified manifest CMV infections to evaluate the role of these viruses in the development of post-transplantation malignancies.

The indications for transplantation were ischemic heart disease (IHD), dilating cardiomyopathy (CMP), valvular insufficiency, and pulmonary hypertension. Among the patients with malignancies, IHD was over-represented. Thirteen (65%) of the 20 tumor patients had undergone heart transplantation for complication of IHD, whereas in the whole transplant series, the proportion of IHD was significantly lower (38%). The proportion of IHD was especially high in patients who developed epithelial malignancies (80%). Overall, among the patients transplanted for IHD, 14% developed post-transplantation malignancies.

The mean age during transplantation of those who developed epithelial malignancies (mean 52.5 yr) was significantly higher than the mean age in the whole transplant series (mean 42.2 yr). The mean age of those who had undergone transplantation for IHD (52.8 yr) was also higher than the mean age in the whole transplant series. The mean age during transplantation of the patients subsequently developing lymphomas was 46.5 yr.

The interval from transplantation to detection of malignancy varied from 2 to 84 months. The mean interval both in the patients with epithelial malignancies and in those with lymphoproliferative disease was 4 yr. Rejection episodes were not more common in the patients with malignancies: 20% compared to 28% in the entire series.

To evaluate the role of EBV in the development of post-transplantation malignancies, the tumor tissues were studied by using in situ hybridisation for Epstein-Barr-encoded RNA (EBER). Intracellular EBV RNA was demonstrated in the two Burkitt's lymphomas and in one diffuse large cell lymphoma (Fig V). The EBER ISH assay worked beautifully with these rather old, paraffin embedded tissue samples; negative and positive control probes were used with on each sample to confirm the preservation of cellular RNA.

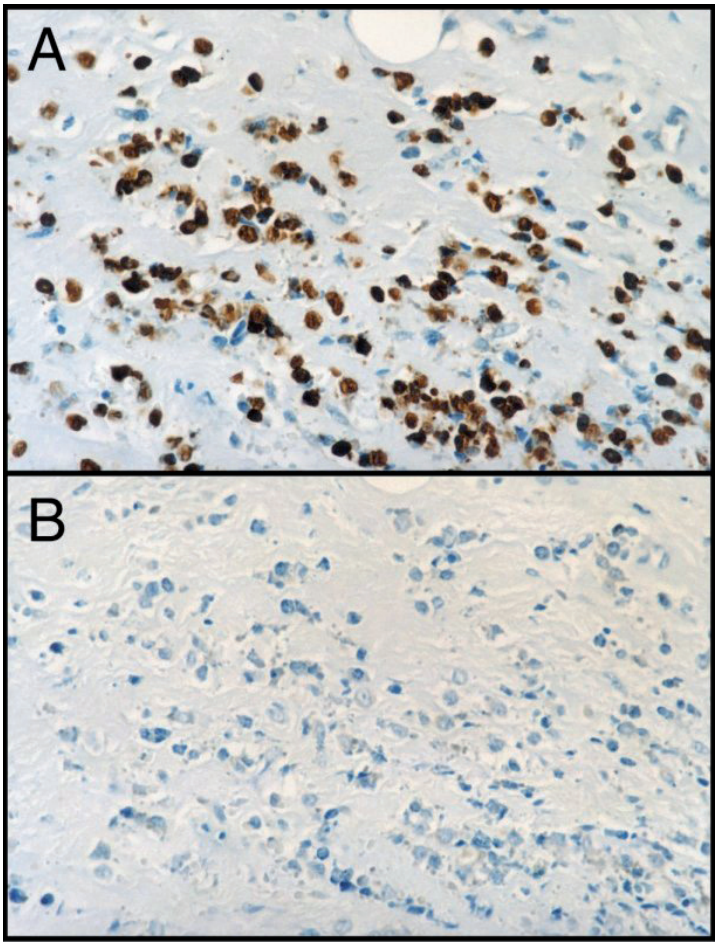

Figure V. Post-transplantation Burkitt's lymphoma (patient 19) studied by in situ hybridisation for EBER (A). The same tissue section hybridised with a negative control probe (B). From II, by permission.

Of the three patients with EBV positive lymphomas, two had a history of post-transplantation CMV infection as opposed to only two of the 14 patients with epithelial malignancies. The frequency of post-transplantation CMV infection in the whole transplant series was 15%, lower than among the patients who developed lymphomas (3/6) and lower than in those who developed EBV-positive lymphomas (2/3). Eight epithelial tumors were screened for HPV DNA, and one was positive. It was an epidermoid carcinoma of the tonsil harbouring HPV type 16.

These results confirm the presence of EBV in lymphomas of heart transplant recipients and suggest that CMV migh have a contributory role in the development of EBV-associated lymphomas.

9.3 LYMPHOPROLIFERATIVE DISEASE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION (III)

We determined the incidence of fatal PTLD among 257 allogeneic stem cell transplantations performed in Helsinki, Finland during 1994-1999. We also assessed the suitability of a quantitative EBV-DNA-PCR for pre-emptive diagnosis of post-SCT PTLD by using ordinary serum samples.

Of the 257 SCT, 132 (51%) recipients were alive and 123 (49%) succumbed by June 2001. The necropsies were analyzed for EBV-related PTLD and the post-mortem analysis revealed 18 cases of PTLD presenting with disseminated infiltrations of EBV-positive lymphocytes. PCR for immunoglobulin gene rearrangement verified clonal proliferation of B-cells in most (59%) cases. The overall incidence of PTLD was 7%; for sibling transplantations 3%, for transplantations with an unrelated donor 14%, and for the deceased, 14%. None of the sibling-graft recipients without aggressive treatment for acute GVHD developed PTLD. Conversely, all the PTLD patients with a sibling donor had undergone intense immunosuppressive treatment for severe acute GVHD, including ATG. All the PTLD patients with an unrelated donor had received ATG. All the 12 PTLD patients with an unrelated donor had received ATG as part of conditioning before transplantation, and five for treatment of severe acute GVHD. In all, the incidence of PTLD was as high as 40% (6/15) among SCT recipients having required intensive GVHD treatment.

Among the 18 PTLD patients, a subset of 12 from whom 103 serum samples obtained sequentially between transplantation and death were available, and were studied further for EBV antibodies and DNA. Before transplantation none of the patients had EBV-IgM, and 11/12 had EBV IgG of high avidity indicating past immunity. The other patient remained EBV-IgG seronegative throughout

follow-up. Post-transplantation, EBV-IgM or heterophile antibodies were not seen, and only one patient presented with a diagnostic rise of EBV-IgG, with constantly high avidity. All in all, EBV serology was of little value in PTLD diagnosis. One the other hand, EBV-DNA in serum became detectable during follow-up of each PTLD patient. The viral DNA was first seen 70 days (median; range 24-154) after transplantation, and 23 (4-86) days before death. The EBV-DNA copy numbers rose progressively to levels as high as 10^6-10^9 per ml serum in PTLD patients sampled shortly before death (Fig. VI). By comparison, fever, the first PTLD clinical symptom appeared 15 days (2-85) before death. All the 15 samples drawn after clinical onset (Fig. VI) were positive for EBV-DNA, corresponding to a qPCR diagnostic sensitivity of 100%. Considering the notable variation in the sample intervals, EBV-DNA appeared to be detectable even before onset in all the patients with appropriate sampling (Fig. VII). Thirty-six SCT recipients were chosen as controls on account of GVHD of similar grade as in the cases; 13 of the donors were siblings and 23 were unrelated. Among the controls, EBV-DNA was detectable in 14/364 (3.9%) sera, corresponding to a diagnostic specificity of 96%. At follow-up, the EBV-DNA positivity of the controls, unlike that of the cases, was confined within isolated samples, and the EBV DNA copy number levels remained below 21 x 10^3 per ml (data not given).

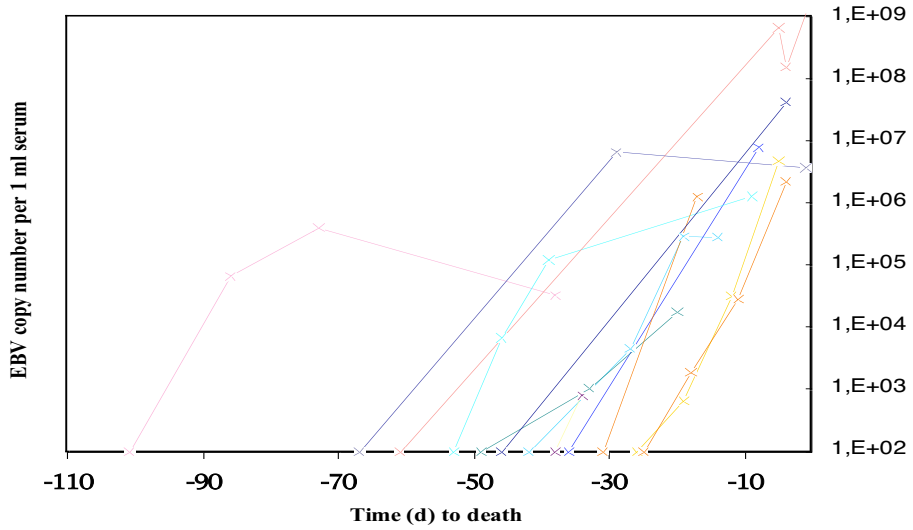

Figure VI. Time course of EBV genome loads measured by real-time PCR in consecutive sera from 12 patients who succumbed from PTLD. Each line indicates the viral load (DNA copies per 1 ml serum) of an individual patient. From III, by permission.

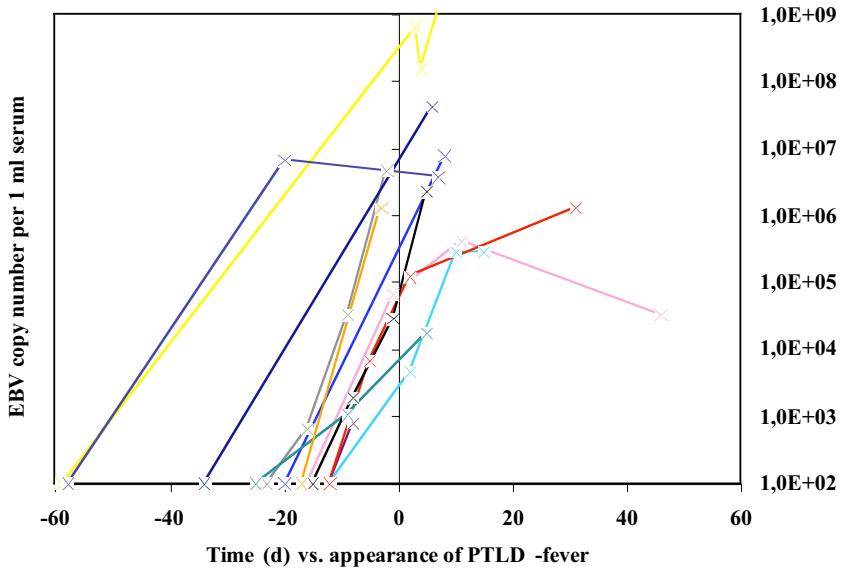

Figure VII. The same EBV genome loads as in Fig.VI, plotted relative to the onset of PTLD-fever in each patient (marked as day 0). From III, by permission.

9.3.1 ANALYSIS OF RISK FACTORS FOR POST-SCT PTLD (IV)

The relation between the occurrence of post-SCT PTLD and the intensity of the immunosuppressive treatment was further studied, to identify high-risk patient groups.

Of the 257 SCT patients, 133 (52%) were alive by June 2001 with a median follow-up time of 1490 days (556–2711), and 124 (48%) had died with a median follow-up time of 274 days (4–1661). The cause of death was transplant-related in 76 patients (61%), relapse in 42 patients (34%), and other in seven cases (5%). In all, 77 (62%) of the 124 deceased patients underwent autopsy. The analysis of the post-mortem reports and the re-evaluation of the autopsy materials revealed or confirmed 19 cases of histopathologically verified PTLD, each presenting with disseminated, multiorgan infiltration of lymphocytes (Table 1). According to the WHO classification, PTLD was monomorphic in 12 patients, polymorphic in six patients, and Hodgkin's-like in one case. Of all

patients, ten showed monoclonality by immunoglobulin heavy-chain rearrangement as detected by PCR.

Table 1 Clinical, laboratory, and autopsy data of 19 patients with PTLD

Number of patients	*Days from transplantation to death*	*Lymphadenopathy during life or at autopsy*	*Diffuse lymphocyte infiltrations at autopsy*	*Histological confirmation of EBV-PTLD*		*LDH U/L*[a] *maximum*	*EBV-DNA copies/ml maximum*[b]	*Diagnosis of PTLD during life*
				EBER	EBNA/LMP			
1	70	No	Yes	+	–	3890	1 290 050	No
2	109	No	Yes	+	+	8410	17 650	No
3	71	Yes	Yes	+	+	3929	1 090 100 000	Yes
4	78	Yes	Yes	+	+	1275	2 238 200	No
5	90	No	Yes	+	+	11790	1 265 300	Yes
6	105	No	Yes	+	–	5246	42 370 000	Yes
7	50	No	Yes	+	+	3750	4 727 900	No
8	235	Yes	Yes	+	+	ND	ND	No
9	145	Yes	Yes	+	+	2769	404 300	Yes
10	188	No	Yes	+	+	1312	ND	No
11	79	Yes	Yes	+	+	1089	ND	No
12	101	No	Yes	+	+	7540	282 150	No
13	67	Yes	Yes	+	+	9380	7 808 750	No
14	127	No	Yes	+	+	2414	6 573 000	No
15	221	Yes	Yes	ND	+	1882	ND	Yes
16	88	Yes	Yes	+	+	1863	2 500 000	Yes
17	763/126[c]	No	Yes	+	+	2899	ND	No
18	292/169[c]	No	No	+	+	2142	ND	No
19	352/89[c]	Yes	Yes	+	+	3247	8 840 000	Yes

All except patient 17 had unexplained fever before death.
[a]Lactate dehydrogenase, normal < 450 U/L.
[b]Detection limit 500 copies/ml.
[c]Days from transplantation/days from DLI.

In 14 cases, PTLD had been diagnosed before the present analysis. In seven of them, PTLD had been diagnosed while the patients were alive based on the histology and positive EBV staining of lymph node and kidney biopsies (LMP) (Patient No. 9) or liver biopsy (LMP) (No. 15), the appearance of EBV positive (EBER and EBNA) and CD20 positive atypical lymphocytes in circulation (Nos. 5 and 6), or the presence of high copy numbers of EBV-DNA in plasma by PCR (Nos. 3, 16, and 19). After death, autopsy confirmed the diagnosis of PTLD in all of these patients (Table 1, study IV). In addition, PTLD was diagnosed in seven more patients at autopsy (Table 1, study IV). In five cases, PTLD was diagnosed as a result of the present study. PTLD was not diagnosed in any of the survivors.

Six of the 19 patients with PTLD had been transplanted from a sibling donor and the remaining 13 patients from an unrelated donor. One PTLD patient with an unrelated donor had received nonmyeloablative conditioning (fludarabine+TBI), and she was also the only PTLD patient having received a blood stem cell graft. All other PTLD patients had been transplanted with a bone marrow graft after myeloablative conditioning. In three patients with an unrelated donor, PTLD occurred

after graft-versus-host disease (GVHD) induced by donor lymphocyte infusion (DLI) given for post-transplant relapse.

In this re-analysis, confirmed PTLD occurred in 19 of the total of 257 patients (7.4%), in 19 of the 124 deceased patients (15.3%), in 13 of the 84 patients with an unrelated donor (15.5%), and in six of the 173 patients with a sibling donor (3.5%). In 16 patients the onset of PTLD was early, and the patients died at a median of 96 days (range 67–221 days) from transplantation. In the remaining three patients, DLI with subsequent GVHD and its treatment was apparently the precipitating event for PTLD.

9.3.2 CLINICAL PICTURE OF POST-SCT PTLD (IV)

The clinical and autopsy findings of the patients with PTLD are shown in Table 1 (study IV). The clinical picture of PTLD lacked distinctive features. With one exception, all patients had fever which appeared at a median of 72 days (29–162) after transplantation (or DLI), and at a median of 16 days (3–155) before death. During life only four patients had palpable lymphadenopathy, and in addition two patients had lymphadenopathy diagnosed by computerized tomographic scan. At autopsy, only six patients had enlarged lymph nodes, although all patients had multiorgan lymphocyte infiltrations. In all, 11 patients showed reactive lymphocytes in circulation. The lactate dehydrogenase concentration was elevated with a rapid increase before death in all patients studied (18/19). Sera were available for EBV-PCR analysis from 13 patients and high copy numbers of EBV- DNA were detectable in all of them at the onset of the symptoms and signs of PTLD. Before transplantation, none of the 13 patients had EBV-IgM and all patients except one (No. 7) had EBV-IgG in their sera, indicating past immunity.

For PTLD treatment three patients (Nos. 5, 6, and 16) received DLI, two patients (Nos. 3 and 19) were treated with one infusion of Rituximab, and one patient (No. 15) with local irradiation and one course of cytotoxic treatment (CHOP). The response was poor in all patients.

9.3.3 THE ROLE OF IMMUNOSUPPRESSION IN RELATION WITH PTLD (IV)

We noticed that all patients with PTLD had been treated with antithymocyte globulin (ATG) either before transplantation, as part of conditioning (unrelated donor), or after transplantation for GVHD, or both. Of the 257 patients, 64 had grade II–IV acute GVHD and were treated with high-dose methylprednisolone (MP). In 30 patients the response to MP was poor, and the patients were given ATG as the second-line treatment. Of the 36 patients given donor lymphocyte infusions (DLI), 11

had grade II-IV acute GVHD after treatment. They were given varying doses of methylprednisolone (MP) and only two patients with an unrelated donor received ATG as second-line treatment.

Among the 173 transplantations from a sibling donor, PTLD was diagnosed in six patients. They all had experienced grade II–IV steroid-resistant acute GVHD and had been treated with ATG. By contrast, among the 158 sibling donor recipients who had not been given ATG, none had PTLD diagnosis. Of the 84 patients transplanted from an unrelated donor with ATG as part of the conditioning, 13 developed PTLD. In nine PTLD patients, ATG had been given only before transplantation. Three of the four patients given Thymoglobuline® 10 mg/kg/day and five of the 26 patients given Thymoglobuline 4 mg/kg/day on three days pretransplantation developed PTLD. Eight of the 13 PTLD patients had not been treated for acute GVHD, one patient had had acute GVHD grade II treated only with MP, and one PTLD patient had had steroid-resistant acute GVHD treated with ATG. In addition, PTLD occurred in three of the 11 patients transplanted from an unrelated donor who had post-transplant relapse and were treated with DLI. All the three patients experienced grade II–IV acute GVHD induced by DLI and two of them were treated with ATG. The use of ATG was a statistically significant risk factor for PTLD ($P<0.0001$), and the PTLD/ATG correlation also remained significant when analysed separately in the groups with or without GVHD.

In conclusion, following stem cell transplantations from an HLA-identical donor with a non-T-cell depleted graft, the risk of PTLD correlated strongly with the intensity of the immunosuppressive treatment. Intensive immunosuppression is a central factor in the development of PTLD, and in this material, the relation between the PTLD and ATG treatment was found to be very close. None of the patients who did not receive ATG developed PTLD.

9.4 EBV INFECTIONS AND LIVER TRANSPLANTATION (V)

The goal of the next study was to clarify the incidence and clinical significance of EBV infections after liver transplantation in Finland. Altogether, 105 liver transplant recipients during years 1999-2002 were sequentially monitored for CMV, HHV-6 and HHV-7 during the first year after transplantation, and the plasma samples were retrospectively studied for EBV-DNA by qPCR. Only 4% (49/1284) of the plasma specimens were positive for EBV-DNA. EBV-DNA was detected in fourteen patients (13%) during the first 12 months. Most patients had EBV-DNA concurrently with CMV (71%) or together with HHV-6 (79%); however only one third of them (29%) had a diagnosis of all three betaherpesviruses. Most patients had EBV-DNA within the first three months after liver transplantation. All examples of low-level EBV-DNA positivity subsided quickly. The peak viral loads of 13 patients were relatively low (median 2100 EBV-DNA copies/ml plasma, range 568-6600) and EBV-DNA usually disappeared within a few weeks (Figure VIII). No clinical signs or symptoms could be attributed to the EBV-DNAemias. Thus, all these EBV episodes of low viral load were transient and clinically harmless reactivations.

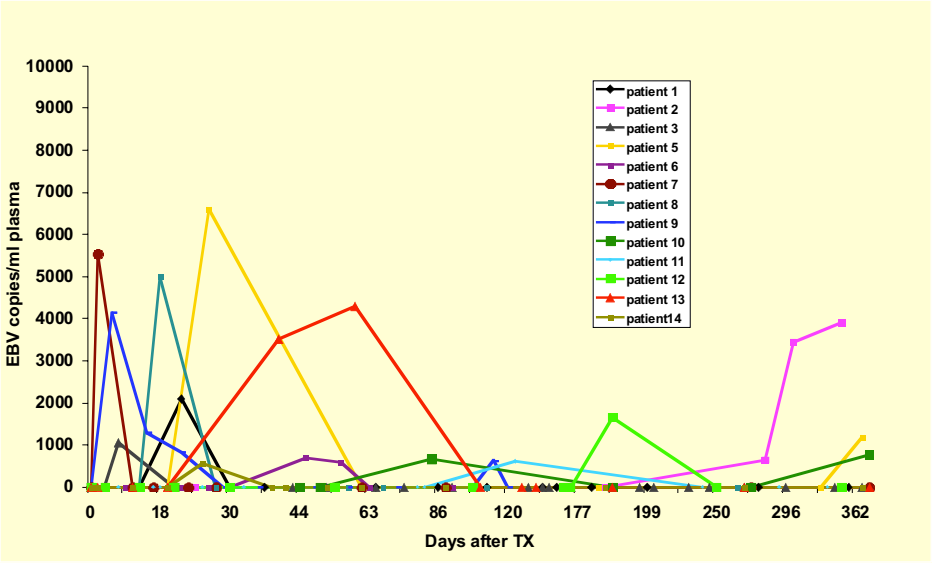

Figure VIII. Viral loads in plasma of the 13 liver transplant recipients with low copy numbers of EBV-DNA. From V, by permission.

9.4.1 PTLD AFTER LIVER TRANSPLANTATION (V)

Although most EBV reactivations were harmless, PTLD was diagnosed in one patient. This patient showed low-level EBV-DNA positivity on day 22 after transplantation, was negative thereafter until day 49, and again was weakly positive up to day 70. On day 175 he suddenly developed a new episode with high copy numbers continuing for six months (median 28 000 copies/ml, range 9851-86 975 copies/ml). None of the other herpesviruses were activated during that time.

Clinical course of PTLD: In 2002 a liver transplantation was performed in a 49-year-old man with liver cirrhosis. CMV infection and a partial thrombosis of the portal vein delayed the post-transplant recovery. Six months after transplantation the patient became jaundice and a CT scan revealed an infiltrative, neoplastic lesion in the head of the pancreas with dilatation of the bile ducts. Histological biopsy showed PTLD, comprised predominantly of B-cells with severe proliferation. The tumor was positive for EBNA by immunohistochemistry and for EBER by in situ hybridisation. The immunosuppressive therapy except corticosteroids was interrupted. The patient received five doses of Mabthera® (a monoclonal antibody against the CD20 antigen) without a clinical response. Thereafter, radiation therapy and cytotoxins were given. The therapies were interrupted for serious gastrointestinal bleedings from the tumor and septicemia. The patient died six months after the diagnosis of PTLD.

In conclusion, concerning liver transplantations, EBV PTLD is a rare but hazardous disease. Low-level EBV DNAemia is often harmless, temporary and concurrent with other beetaherpesviruses. Also in liver recipients EBV qPCR seems to be crucial for PTLD diagnosis. During PTLD treatment sequential monitoring of EBV DNA levels appears important and can be used to guide therapy decisions.

9.5 ANALYSIS OF 406 SCT RECIPIENTS WITH EBV qPCR (VI)

Altogether, 57 of 406 patients (14.0%) showed EBV DNA in at least one serum sample. The total number of positive samples was 105 (1.9%) out of the 5479 samples studied.

Group I: Twenty two patients (5.4%) showed high (median 40 million) copy numbers of EBV DNA. In all 22 patients the EBV copy numbers rose progressively, with peak levels occurring shortly before death (14 patients), or the count was already high in the first positive sample (eight patients). In the first positive sample of all 22 recipients, the median EBV-DNA copy number was 32 million, range 600-660 million. The difference between the last negative and the first positive sample was 5-52 days (median 21 days). The median count in the last sample was 54 million (median; range 50 100-1090 million); these last samples were taken at a median of 8 days (range 1 to 61 days) preceding death. The viral DNA was first detected at a median of 63 days (range 24 to 330 days) after transplantation, or at a median of 18 days (range 4 to 84 days) before death (Figures IX and X). In one relapsed patient the EBV DNA appeared at ten months post-transplantation following donor lymphocyte infusion-induced GVHD. All 22 patients died, and the diagnosis of EBV-PTLD could be confirmed in autopsy in 15 cases showing disseminated infiltrates of EBV-RNA containing lymphocytes.

Group II: In addition, 16 patients (3.9%) showed a positive EBV-PCR finding with a low copy number, median 5370 (range 800 - 17 650) observable in only one or two samples before death (15 patients) or in the last sample available (one patient alive) (Table 4 & Figure IX). These terminal samples had been collected at a median of 94 days (range 18 – 534 days) after transplantation, or at a median of 69 days (range 3 – 444 days) before death. Five of these patients showed histologically confirmed PTLD in the post-mortem study. The last serum samples of those five patients were taken at a median of 80 days (range 4-230 days) before death. The other causes of death were GVHD (six patients), other infections (one), relapses (two) and veno-occlusive disease (one).

Table 4. EBV qPCR findings of the last samples in 16 patients with low terminal EBV-qPCR finding in relation to time (mo) after transplantation and time (mo) before death. From VI, by permission.

n	Time (mo) after transplantation	Time (mo) to death	EBV DNA count	Death cause
1	0,6	0,1	7700	VOD
2	8,5	14,6	3220	Relapse
3	5,0	1,3	8910	Relapse
4	2,0	3,9	4190	**PTLD**
5	2,9	1,6	3080	Infection
6	2,0	0,2	1270	GVHD
7	2,0	7,7	3410	**PTLD**
8	2,5	0,7	8340	GVHD
9	5,1	1,1	800	**PTLD**
10	17,9	alive	2060	
11	2,0	0,4	1355	GVHD
12	2,2	0,2	4990	GVHD
13	2,0	0,9	800	GVHD
14	2,4	0,1	15000	**PTLD**
15	3,0	0,8	3200	GVHD
16	3,0	0,7	17650	**PTLD**

Group III: Nineteen patients (4.7%) showed in serum transient EBV DNA positivity. At follow-up, ten of these 19 patients had only one sample containing EBV-DNA (the initial positive sample); seven patients had two positive samples, and two had three positive samples. The viral DNA was first seen at a median of 98 days (range 24 – 537 days) after transplantation. The EBV DNA copy numbers (median 6300; range 700 - 36 200) remained <50 000 in all patients. During follow-up none of these patients was known to develop PTLD. Of these 19 patients seven are still alive.

Group IV, all the remaining patients: All in all, 349 patients (86%) had no detectable EBV DNA in serum. During follow-up only two of these patients were known to develop PTLD, beyond sampling for this study, following relapse and DLI-induced severe GVHD.

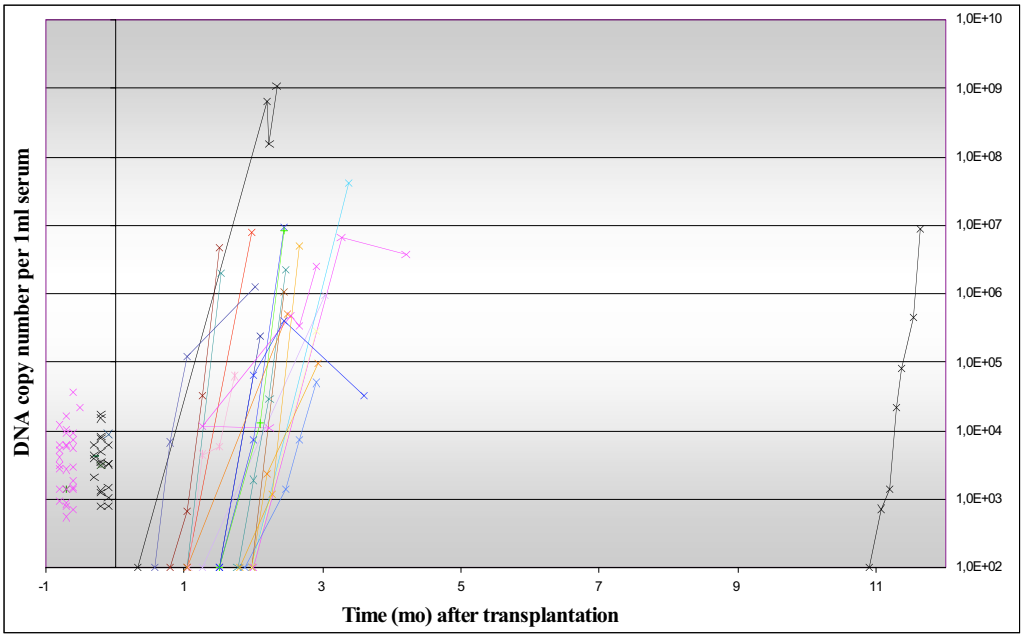

Figure IX. Follow-up of the patients with fatal EBV infection; each line represents one patient. The levels of EBV DNA in patients with transient EBV infection or low terminal copy numbers are shown on the left; in pink and in black, respectively. From VI, by permission.

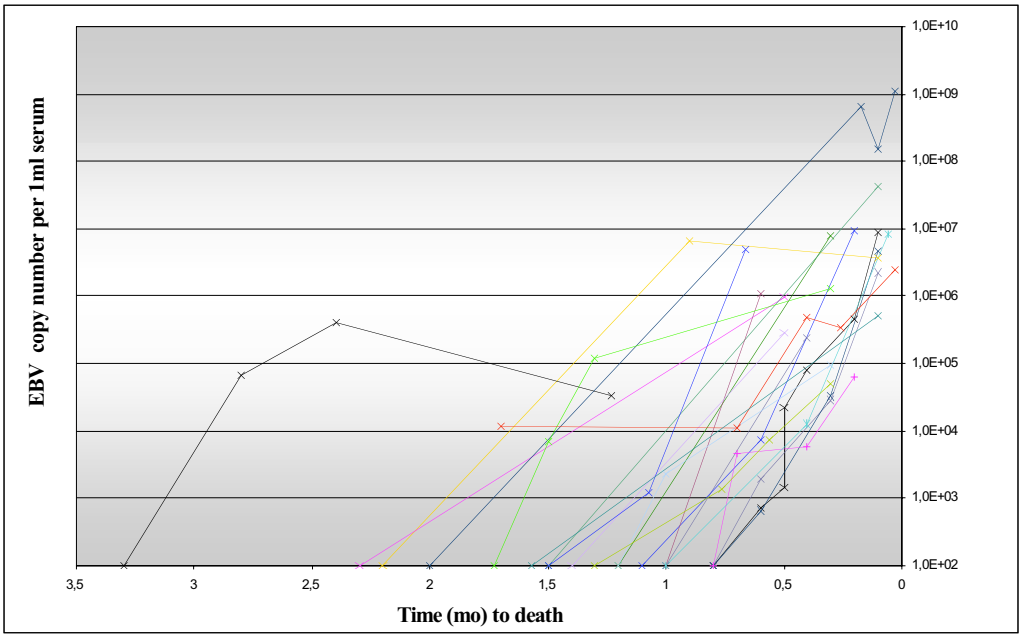

Figure X. The same EBV DNA loads as in Figure IX, plotted relative to death (marked with 0). From VI, by permission.

9.5.1 AN ALGORITHM TO DISTINGUISH BETWEEN SEVERE AND TRANSIENT EBV INFECTION (VI)

We compared the EBV qPCR data of patients with fatal or transient EBV infection, and we created an algorithm for prediction of the severity of EBV infection. As mentioned above, 22/406 (5.4%) patients with fatal EBV-infection showed high (median 40 million) copy numbers of EBV DNA. In all 22 patients the EBV copy numbers rose progressively or the count was already high in the first positive sample. Transient EBV DNA positivity was seen in 19/406 (4.7%) patients. By comparing the amount of EBV DNA and the rapidity of the increase in the EBV DNA levels, these two groups of patients could be distinguished: The first criterion is the EBV DNA level in the first positive sample: low (<50 000 copies/ml) or high (≥50 000 copies/ml). If this EBV DNA level is ≥50 000, the patient can be classified as having life-threatening EBV infection. On the other hand, if this initial EBV DNA level is <50 000, two possibilities exist: (i) The subsequent sample is EBV-qPCR negative. Conclusion: the patient does not have life-threatening EBV infection. (ii) The subsequent sample(s) is EBV-qPCR positive. Then, the rapidity of the increase in the DNA levels is considered, according to the mean value of the derivatives of the log-transformed EBV DNA values. If this value exceeds 3.6, the patient is at a high risk of having life-threatening EBV infection (Figure XI).

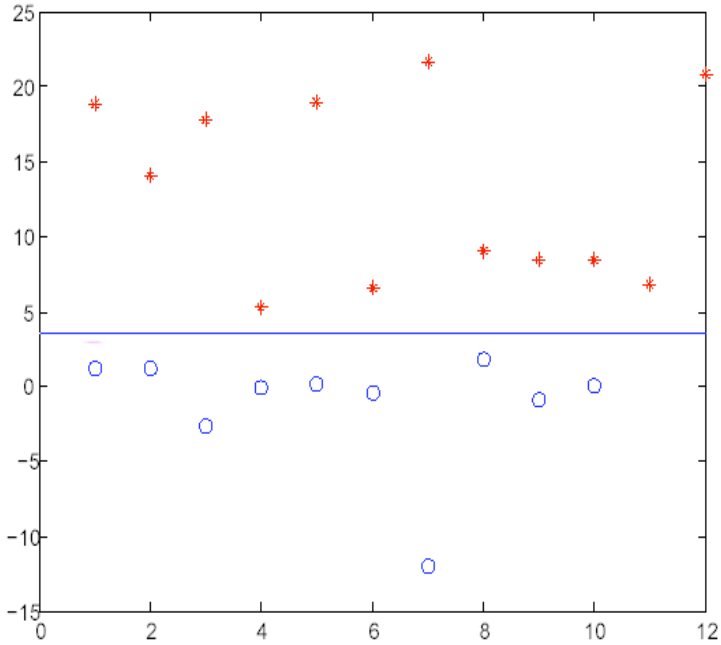

Figure XI. An algorithm was created by using the EBV PCR data of groups I and III for distinction of the patients who had fatal EBV infection from those who had only transient EBV infection. The patients with EBV infection of fatal outcome (red asterisks) fall above the cut-off, and those with spontaneously resolving EBV infection (blue circles), below it. From VI, by permission.

In conclusion, low-level EBV-DNA positivity in serum occurs relatively frequently after stem-cell transplantation and may subside without specific treatment. However, high molecular copy numbers (>50 000) predict the development of PTLD very strongly, are not spontaneously reversible, and warrant immediate treatment. Moreover, for identification of the patients with severe EBV infection, we created an algorithm which distinguished all the patients with a fatal EBV infection from those with a transient EBV reactivation. In the former patients unlike in the latter, with the EBV DNA levels rising rapidly, the mean value of the derivatives of the log-transformed DNA values exceeded the cut-off value of 3.6, cautioning against life-threatening EBV infection.

10. DISCUSSION

10.1 CMV INDUCED EBV IMMUNOREACTIVATION (I)

We demonstrated with widely used EBV antibody assays that a large proportion of patients with CMV primary infection showed antibody profiles of EBV recurrence: seroconversion of VCA IgM and/or ≥ fourfold rise of VCA IgG, together with high (or intermediate) avidity of EBV IgG. Immunocompetent individuals and transplant recipients differed in the frequency of CMV-associated EBV serodiagnosis (17% and 50%, respectively). In contrast, EBV primary infection reciprocally did not appear to induce immunoreactivation of CMV.

In the literature, to date no single commonly accepted serological criteria exist for EBV reactivation. Many different parameters have been used: rise of EA IgG or EA IgA titer (Hornef et al., 1995), seroconversion of EA IgM (Hornef et al., 1995), decrease of EBNA IgG (Quesnal et al., 1992; Taneichi et al., 1993; Hornef et al., 1995), presence of VCA IgM (Hornef et al., 1995), increase of VCA IgG (Rahman et al., 1991; Quesnal et al., 1992; Glaser et al., 1994), or simultaneous positivity to EA IgM and EBNA IgG (Obel et al., 1996). The ZEBRA protein (BamHI Z EBV replication activator) controls the switch of EBV from a latent to a productive cycle (Countryman et al., 1985 and 1987; Chevallier-Greco et al., 1986; Lieberman et al., 1986), and ZEBRA IgG has been proposed as a serological marker for EBV reactivation (Maréchal et al., 1993). On the other hand ZEBRA antibodies in healthy EBV-seropositives are rarely detectable, they not uncommonly (75-87%) occur in patients with nasopharyngeal carcinoma, but also during infectious mononucleosis (85%) (Joab et al., 1991; Mathew et al., 1994). In this study we chose strict serological criteria for EBV reactivation: seroconversion of VCA IgM and/or ≥ fourfold rise of VCA IgG, together with high or intermediate avidity of VCA IgG.

In theory, rising levels of EBV antibodies in CMV primary infection could be due to polyclonal B-cell stimulation, to antibody cross-reactivity, or to selective stimulation of memory B-cells, either with or without virus replication due to endogeneous reactivation or exogenous reinfection. Polyclonal antibody rises have been found long ago in CMV primary infection (Klemola et al., 1969), but these are usually of the IgM class, in contrast with the extensive titer rises of high-avidity VCA IgG found in many of our patients. Moreover, rising IgG for varicella zoster virus,

another latently infecting herpes virus, was observed in only one of our CMV patients, which further argues against general immunoactivation as a cause of our findings. It is unlikely that, antigenic cross-reactivity between the betaherpesvirus CMV and the distantly related gammaherpesvirus EBV would explain the high antibody titer rises; absorption studies have shown no such cross-reactivity (Linde et al., 1990).

The vast majority of the patients with CMV-induced EBV immunoactivation also showed immunoactivation for HHV-6. However, the specificity of the HHV-6 antibody rises is less clear-cut than that of the EBV serodiagnoses. CMV and HHV-6 show high sequence similarity (Efstathiou et al., 1988; Chou et al., 1992), and immunological cross-reactivities between these two viruses have been detected in some but not all antibody adsorption studies (Irving et al., 1990; Sutherland et al., 1991; Ward et al., 1991 and 1993). Kinetic differences have suggested that HHV-6 antibody rises would be at least partially virus species-specific (Irving et al., 1990). In vitro virus replication studies (Flamand et al., 1993) have also supported the possibility that EBV could serve as a stimulatory cofactor for the induction of HHV-6 or vice versa.

Evidence is accumulating on virus-virus interactions in vivo. EBV reactivation by human immunodeficiency virus (HIV) has been documented by appearance of EBV particles in blood and secretions (Birx et al., 1986; Alsip et al., 1988; Diaz-Mitoma et al., 1990) and by the progressive increase of VCA IgG in relation to a decrease in CD4 cell count (Quesnal et al., 1992). Findings suggestive of EBV activation (coexpression of EA IgM and EBNA IgG) have been observed in apparently immunocompetent (Färber et al., 1993) or immunosuppressed (Hornef et al., 1995) patients with CMV infection. Our data provide the first unequivocal evidence for EBV immunoreactivation in a large proportion of patients with CMV primary infection.

Since our work a case report has been published on EBV hepatitis with ascites, and subsequent EBV recurrence during CMV primary infection (Rajwal et al., 2003). Drouet and colleagues (1999) reported signs of CMV and EBV coinfections in graft recipients and speculated that CMV could stimulate EBV replication and dissemination. Earlier, Meyer and colleagues (1996) noted the importance of simultaneous active CMV and EBV infection in renal transplant recipients; severe clinical symptoms were observed only in the group of combined CMV and EBV infections. These authors proposed that simultaneous EBV replication might be an important co-factor for the development of CMV disease.

Our findings make it clear that, with widely used serological procedures a large number of CMV primary infections are being diagnosed as EBV infections. Such errors can have serious consequences during immune suppression or pregnancy, when CMV infections are severe. Furthermore, it can be inferred from these results that the criteria of EBV serodiagnosis need strengthening. Correct identification of the primarily infecting herpes virus requires serological markers stronger than IgM detection or an IgG rise. Consistent with earlier data, our findings showed the usefulness of IgG avidity determination in pointing out the primary infectious agent.

10.2 ROLE OF VIRUSES IN THE DEVELOPMENT OF MALIGNANCIES AFTER HEART TRANSPLANTATION (II)

Six of the 249 patients after heart transplantation developed lymphomas, of which three were positive for EBV RNA. This is in line with previous observations of the overrepresentation of EBV positivity in post-transplantation lymphoproliferative disorders of the B-cell phenotype (Swerdlow, 1992; Boubenider et al., 1997; Liebowitz, 1998; Penn, 1999). Clinical CMV infection appeared to occur more frequently in the patients who later developed EBV-positive lymphoma (2 of 3) than in the patients who developed epithelial malignancies (2 of 14) and in the patients of the whole transplant series (15%) (Mattila et al., 1997). This is interesting in the context of the oncogenic potential of CMV *in vitro* (Raza, 1998; Cinatl et al., 1996; Boldogh et al., 1994). On the other hand, as we concluded above, CMV infections may reactivate EBV in vivo (Hornef et al., 1995; study I), which in immunosuppressed patients could possibly predispose to B-cell proliferation. However, it is possible that both CMV disease and the EBV-positive lymphomas in our series merely had a common, immunosuppression-related cause. Previously, the risk of non-Hodgkin's lymphomas after transplantation has been attributed to the aggressiveness of the immunosuppressive regimen (Opelz et al., 1993).

The average follow-up time of the 20 patients who developed malignancies was 6.5 yr. The annual risk for cancer was 1.2%, and excluding skin tumors (basal cell carcinoma and Morbus Bowen of the skin), it was 1.0%. The incidence of new cancers (excluding basal cell carcinoma) in the Finnish population of 5 million is 8900 (0.18%) a year. It thereby appears that the likelihood of malignancy development after heart transplantation is severalfold higher than in the general population. The incidence of cancer after heart transplantation in our material appears to be comparable to that previously reported for heart and kidney recipients (Birkeland et al., 1995; Curtil et al., 1997).

EBV in situ hybridisation (ISH) turned out to be an excellent method for detecting EBV in paraffin-embedded – even old – tissue sections. The EBER transcripts are abundantly expressed in latency (Arrand et al., 1982; Howe et al., 1986; MacMahon et al., 1994; Nanbo et al., 2002). With EBER ISH it is easy to localise the EBV containing cells in clinical tissue samples. With this ISH method, EBV has been localised to the Reed-Sternberg cells in Hodgkin's disease (Wu et al., 1990). The sensitivity of EBER detection has allowed for the detection of EBV not only in Reed-Sternberg and Hodgkin's cells, but also occasionally in small benign-appearing cells in tumour tissue (Weiss et al., 1991; Herbst et al., 1992; Khan et al., 1992). EBER ISH works well also in PTLD diagnosis if the suitable tissue samples are available. Oral hairy leukoplakia and some hepatocellular carcinomas are the exceptions, in which EBERs are not expressed (Gilligan et al., 1990; Niedobitek et al., 1991; Ryon et al., 1993, Sugawara et al., 1999). The specificity of the EBER ISH technique has been confirmed by the lack of any background signal or cross-hybridisation in clinical tissues (MacMahon et al., 1991; MacMahon et al., 1994). The EBERs are consistently expressed in EBV-associated tumors regardless of the pattern of latent gene expression (MacMahon et al., 1994; Nanbo et al. 2000).

Of note, cyclosporin A (CyA) induces immunosuppression by inhibiting the transcription of several cytokines including interleukins 2 and 4 and gamma interferon by disrupting calcium-mediated intracellular signals in T lymphocytes upon forming a complex with cyclophilin (Bierer et al., 1990; Mattila et al., 1990), which inactivates the protein phosphatase calcineurin (Friedman et al., 1991; Liu et al., 1991). The resulting immunosuppression may lead to an increased risk of lymphoproliferative disease by impairing immunity to lymphotropic viruses such as EBV. Another mechanism that might contribute to the development of malignancies during CyA treatment is enhanced production of transforming growth factor beta (Li et al., 1991; Prashar et al., 1995), which has been shown to enhance tumor growth in animal models (Hojo et al., 1999). Other immunosuppressive drugs that are carcinogenic, azathioprine and cyclophosphamide, may also contribute to the increased incidence of cancer among transplantation patients. Careful adjustement of the immunosuppressive treatment can be expected to have a beneficial effect on prevention of post-transplantation malignancies.

10.3 LYMPHOPROLIFERATIVE DISEASE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION (III)

During 1994-1999 altogether 257 adult patients underwent allogeneic stem cell transplantation (SCT) at Helsinki University Central Hospital. We determined by strict histopathological criteria the incidence of fatal Epstein-Barr virus (EBV) – related PTLD, and set up a quantitative EBV-DNA-PCR - to our knowledge the first quantitative "real-time" PCR assay in Finland for any microbial pathogen - and assessed its diagnostic value by using ordinary serum samples. Our study showed that the risk of developing PTLD is strongly associated with the type and intensity of immunosuppressive treatment. None of the sibling-graft recipients who had not required aggressive treatment for acute GVHD developed PTLD. However, the incidence of this disease was 14% among the recipients from an unrelated donor (who all received ATG prophylactically), and 40% among such recipients that had required ATG for therapy of GVHD. These findings are in line with previous reports showing an increased risk of PTLD due to strong immunosuppressive measures such as T-cell depletion of the graft or administration of ATG or anti-CD3-antibodies (Shapiro et al., 1988; Zutter et al., 1988; Witherspoon et al., 1989; van Esser et al., 2001).

The lymphoproliferations were disseminated, with EBV-positive lymphocytes infiltrating several organs; and most cases were clonal. The onset of PTLD in relation to donor cell administration was early, i.e. within 6 months of SCT or DLI, and disease progression was rapid, in 15 days (range 2-85) from initial symptoms to death. The clinical picture of PTLD lacked distinctive features, making clinical diagnosis impossible. Due to the scarcity of lymph nodes for biopsy, histologic techniques were of little use during the patients' lifetime. Indeed, in the absence of a rapid and reliable diagnostic method, PTLD in this retrospective series was diagnosed too late for treatment to be effective.

All the PTLD patients exhibited EBV-DNA detectable in archived sera by real-time qPCR. The diagnostic performance of this method, in terms of distinction of the PTLD cases from corresponding controls, turned out to be excellent. With respect to EBV-DNA positivity in an individual specimen, the diagnostic sensitivity according to study III was 100%, and also the diagnostic specificity was very high (96%). Such a high specificity is in line with the absence of viral DNA in plasma of solid-organ recipients carrying EBV chronically in memory B-cells (Babcock et al., 2000; Rose et al., 2001). In serial specimens of the cases, but not of the controls,

the EBV-DNA copy numbers rose rapidly and progressively towards approaching death. The magnitude of the extracellular EBV-DNA loads in all our patients suggests that the early-onset infiltrative PTLD involves lytic EBV infection. Similarly (yet not equally) high DNA loads have been encountered in the present and earlier studies in patients with acute EBV primary infection (Gan et al., 1994; Yamamoto et al., 1995; Meerbach et al., 2001; Chan et al., 2001; Brengel-Pesce et al., 2002). Importantly, the appearance of viral DNA in serum preceded the PTLD symptoms, a prerequisite for pre-emptive treatment.

10.3.1 PRE-EMPTIVE DIAGNOSIS OF LYMPHOPROLIFERATIVE DISEASE (III)

Based on our study it is evident that, for pre-emptive PTLD diagnosis among allogeneic stem-cell transplant recipients by using quantitative EBV-DNA detection, serum is a highly recommended sample type. In these patients, cytopenia during the first months is frequent, underlining the diagnostic utility of serum or plasma relative to leukocytes. Several studies among stem-cell recipients have quantified EBV-DNA in samples containing leukocytes (Lucas et al., 1998; Yoshinobu et al., 1999; Gustafsson et al., 2000; Yang et al., 2000; Hoshino et al., 2001; Gärner et al., 2002). Niesters and colleagues (2000), however, observed plasma to be an excellent sample type for real-time PCR diagnosis of SCT-associated PTLD; a finding brifly reported earlier (Beck et al., 1999) and recently confirmed (Berger et al., 2001; Meerbach et al., 2001; Ohga et al., 2001; Brengel-Pesce et al., 2002). Also in solid-organ-PTLD, serum or plasma is suitable for EBV-DNA quantification (Limaye et al., 1999; Lei et al., 2000; Wagner et al., 2001). On the other hand, Stevens and colleagues (2001) stated that in lung transplant recipients with late-onset PTLD, increased EBV DNA loads in blood are restricted to the cellular compartment; a discrepancy warranting further study among the various forms of lymphoproliferative disease.

In conclusion, we have shown that EBV-associated early-onset PTLD is an important cause of mortality also in matched, allogeneic non-T-cell depleted stem-cell transplantations. In light of the rapidity of disease progression and the emergence of efficient therapeutics, regular monitoring of EBV DNA levels in serum or plasma can be strongly recommended for routine practise in profoundly immunosuppressed patients.

10.3.2 ANALYSIS OF RISK FACTORS FOR POST-SCT PTLD (IV)

In the present project, the possible risk factors for the occurance of PTLD were studied in order to identify high-risk patients in view of prophylactic and pre-emptive treatment. During the years 1994-1999, a total of 257 adult patients underwent non-T-cell-depleted allogeneic stem cell

transplantation at Helsinki University Central Hospital. Of the donors, 173 were siblings and 84 unrelated. In 250 cases, the conditioning was myeloablative and in seven cases nonmyeloablative. In most patients the indication for transplantation was a hematological malignancy and in five patients, aplastic anemia.

PTLD in general is a rare complication of allogeneic stem cell transplantation, yet intensive immunosuppression increases its incidence (Zutter et al., 1988; Micallef et al., 1998; Lucas et al., 1998; Curtis et al., 1999; van Esser et al., 2001). Our findings are in agreement with this notion. In our study, 19 cases of PTLD were found among 257 consecutive allogeneic transplantations of non-T-cell-depleted grafts from HLA-identical siblings or unrelated donors. In all cases, PTLD was an early event with the first signs appearing at 29-162 days after the transplantation or donor lymphocyte infusion, the median being 72 days. The incidence 7.4% (19/257) may be an underestimate. The present analysis was retrospective and mainly based on the autopsy material, and only about two-thirds of the succumbed patients were autopsied. No cases of PTLD were diagnosed among the survivors. The present findings may, however, reflect relatively well the actual situation, as the early type of PTLD usually is fatal (Zutter et al., 1988; Benkerrou et al., 1998; van Esser et al., 2001).

The occurrence of PTLD was strongly associated with the degree of immunosuppression. No PTLD developed among the patients having received a graft from a sibling donor without steroid-resistant acute GVHD necessitating treatment with ATG. Of the 55 patients transplanted from an unrelated donor with ATG as part of the conditioning, eight (15%) developed PTLD despite the absence of GVHD. The larger immunological differences in the unrelated donor situation as compared to the sibling donors – despite A, B, DR antigen match – may have contributed to the immunosuppression. Where transplants are performed using graft from an unrelated donor, the addition of low-dose corticosteroid to the GVHD prophylactic treatment consisting of cyclosporine and methotrexate may have been a further contributing factor.

The present study also suggests a difference in the immunosuppressive effect between the different ATG products in the doses used, which is in accordance with the study by Gaber and colleagues (1998). They showed that the degree of T-cell depletion caused by Thymoglobuline® was significantly greater and longer-lasting when compared to Atgam®. Thymoglobuline® was also more effective in the treatment of acute rejection. In our study, the incidence of acute GVHD was higher and the incidence of PTLD lower, among the patients who had been transplanted from an

unrelated donor and given pretransplant Atgam® compared to those who had been given Thymoglobuline®. In the immunosuppressive treatment after stem cell transplantation, the balance between effective GVHD prophylaxis and the complications of immunosuppression is extremely delicate. Since this study, the patient management in transplantation from an unrelated donor has been modified by omitting corticosteroids from the GVHD prophylaxis as well as by reducing the dose of Thymoglobuline®. The incidence of GVHD has somewhat increased, but those of viral infections and PTLD significantly decreased (Volin et al., 2002).

EBV-induced PTLD is a serious and often fatal complication of allogeneic stem cell transplantation, whereby it is of vital importance to identify the high-risk patients for early diagnosis and treatment. Intensive immunosuppression is a central factor in the development of this complication, and in the present material, the relation between the PTLD and ATG treatment was found to be close. None of the present patients who did not receive ATG developed PTLD. Even in those who were given ATG as part of the conditioning and who did not show any acute GVHD, the risk seems to be increased, but the effect of the dose and the type of ATG still need further evaluation. The clinical diagnosis of PTLD is almost impossible, as the symptoms and signs are unspecific. However, quantitative measurement of EBV DNA in serum was in this as well as in other recent studies shown to be a very useful for the prediction and the early diagnosis of PTLD (Kimura et al., 1999; van Esser et al., 2002; Clave et al., 2004; Wagner et al., 2004; study III). The demonstration of rapidly increasing or high levels of EBV-DNA allow early intervention with reduction of immunosuppression and use of specific antibodies (e.g. Rituximab®) or donor lymphocyte infusion.

10.4 EBV INFECTIONS IN ADULT LIVER TRANSPLANTATION (V)

PTLD carries a significant mortality also in solid organ transplantations. Although several publications on EBV viremia in stem cell transplant recipients exist, those after liver transplantation mainly deal with pediatric patients and only a few have been published on adult liver transplant recipients.

Pediatric and adult liver transplantations differ markedly concerning the risk for PTLD. For pediatric patients the PTLD risk is particularly high in EBV primary infection (Newell et al., 1996). Other risk factors are young age and the combination of EBV-positive donor and EBV-negative recipients (Ho et al., 1988; Sokol et al., 1993; Cox et al., 1995; Walker et al., 1995). The incidence of PTLD in pediatric patients varies between 3-13% depending on the immunosuppressive regimen

(Ho et al., 1988; Malatack et al., 1991; Nalesnik et al., 1992; Newell et al., 1996; Jain et al., 2002), whereas adult liver recipients have an overall low incidence of PTLD (0.8-3%) (Nalesnik et al., 1992; Niedobitek et al., 1997; Jain et al., 2002). Monitoring of EBV DNA by quantitative PCR has been shown to be effective for diagnosis of PTLD also among liver transplant recipients (Riddler et al., 1994; Lucas et al., 1998; McDiarmid et al., 1998; Gridelli et al., 2000; Spada et al., 2001; Matsukura et al., 2002).

In our study on adult liver transplant recipients, a low level of EBV-DNA was detected in 13% of the patients. DNA occurred usually shortly after transplantation and together with reactivation of other herpesviruses. EBV-DNA subsided within a few weeks. These EBV-DNA episodes seemed to be clinically nonsymptomatic reactivations. One patient developed PTLD six months after transplantation with increased amounts of EBV-DNA (median 28 000 copies/ml, range 9851-86975). Before development of PTLD this patient had shown low-level and transient episode of EBV-DNA and during PTLD he presented with high levels of EBV DNA. This finding is in disconcordance with Barkholt and colleagues (2005). They had studied adult liver transplant recipients with quantitative EBV PCR and they did not find higher EBV DNA loads in serum of the one patient with PTLD compared with the other patients.

In our study on adult liver transplant recipients, plasma specimens and a real-time TaqMan assay were used to quantify EBV viral loads. EBV-DNA was detected in 13% recipients with viral loads of 568-6600 copies/ml plasma, in line with previous studies (Niesters et al., 2000; Wagner et al. 2001), while the peak viral load in the PTLD patient was 86 975 copies/ml. The same TaqMan-based real-time EBV-DNA PCR turned out to be highly sensitive (100%) and specific (96%) in the diagnosis of severe EBV infection in bone-marrow stem cell recipients (studies III and IV). The technique is fast, user friendly and highly reproducible (Kimura et al., 1999; study III). Furthermore, the MagNA Pure extraction simplifies the DNA extraction for this approach and shortens its hands-on time (Stöcher et al., 2003; Fafi-Kremer et al., 2004). Of note, automated extraction of EBV-DNA is easier to perform with whole blood or plasma than with PBMCs, and facilitates the standardizaton of EBV DNA measurement (Fafi-Kremer et al., 2004). Real-time PCR technology used here was suitable for consecutive screening of EBV DNA levels and could be used in diagnosis and monitoring of EBV-induced lymphoproliferative disease in liver transplant recipients.

In our retrospective study, only one liver recipient showed high viral loads and developed PTLD with poor clinical outcome. In conclusion, although most EBV reactivations were not severe, fatal PTLD was diagnosed in one patient. As far as liver transplantations are concerned, EBV PTLD is a rare but hazardous disease. Low-level EBV DNAemia is often harmless, temporary and concurrent with other betaherpesviruses. Also in liver recipients EBV qPCR seems to be crucial for PTLD diagnosis. During PTLD treatment sequential monitoring of EBV DNA levels is important and can be used to guide therapy decisions.

10.5 SAMPLE MATERIAL FOR EBV qPCR: SERUM OR PBMC?

Quantitative PCR has been successfully performed with peripheral blood mononuclear cells (PBMC), with unfractioned blood and with serum or plasma. As yet no consensus exists as to which is the sample type of choice. All these materials have their pros and cons. When interpreting the EBV DNA levels of different studies we must keep in mind that the DNA values are not comparable because they have not been calibrated with a common standard.

Kimura and colleagues (1999), using PBMC for qPCR, studied 18 patients with symptomatic EBV infections (four chronic active EBV infection, five PTLD, nine patiens with infectious mononucleosis), ten unsymptomatic transplant recipients and 13 immunocompetent patients suspected of having EBV primary infection. The mean load of EBV DNA in the PBMC was $10^{3.7}$ copies/µg of DNA in the patients with PTLD, $10^{4.1}$ copies/µg in the patients with chronic active EBV infection, and $10^{2.2}$ copies/µg in the patients with infectious mononucleosis. These numbers were significantly larger than those for the recipients without EBV diseases or for the immunocompetent controls. However, there was some individual overlap. The authors studied both plasma and PBMC samples from one patient with infectious mononucleosis; the results were quite uniform (Kimura et al., 1999). Tsai and colleagues (2002) reported the sensitivity of qPCR with PBMC being only 39% in adult solid-organ recipients with PTLD. Wagner and colleagues (2004) studied by qPCR the PBMC of SCT recipients and reported 11% with single episodes of high-levels of EBV DNA (>4000 EBV copies/µg PBMC DNA) and 19% with 2 or more such episodes. Altogether 9.4% of the recipients developed PTLD symptoms and were treated with EBV-specific cytotoxic T cells or anti-CD20 antibody. Using PBMC for EBV qPCR Wagner and colleagues (2004) recommended prompt rather than preemptive PTLD treatment.

Stevens and colleagues (2001) suggested in a study on pulmonary transplant recipients that the increased EBV loads in PTLD patients were restricted to the cellular blood compartment. Whole blood includes all compartments that may harbour EBV, and has been claimed to be the best sample type for the absolute EBV burden in circulation, as compared with isolated cell fractions (Leung et al., 2004). Leung and colleagues (2004) studied EBV DNA loads using samples of unfractioned whole blood in renal, hepatic, and cardiothoracic transplant recipients and found that the post-transplant EBV loads were significantly higher than the pre-transplant levels. All recipients had transient rises of EBV –DNA level, whereas that of one patient suspected to have PTLD continued to rise (Leung et al., 2004). Whole blood EBV DNA quantification has been furthermore claimed to be more appropriate for the purpose of advocating either prompt or preemptive treatment among patients who might develop PTLD (Wagner et al., 2004). Scheenstra and colleagues (2004) analysed whole blood samples of pediatric liver transplant recipients with competitive quantitative EBV PCR and noticed that the EBV DNA loads are higher in patients with primary infection than in those who are EBV seropositive before transplantation. In their study two patients who developed PTLD had a high circulating viral load (20 500 copies/ml and 446 000 copies/ml) (Scheenstra et al., 2004).

However, as a sample material, PBMC are difficult to obtain from cytopenic patients; such is often the case when the immune reconstitution is delayed or during the course of Rituximab® therapy (Yang et al., 2000; van Esser et al., 2002; Clave et al., 2004). Clave and colleagues (2004) studied by qPCR both cell and plasma samples obtained after allo-SCT: EBV-DNA was seen in 50% of PBMC and in 16% of samples of plasma, respectively. The patients who showed EBV DNA in plasma displayed also the highest cellular viral loads (Clave et al., 2004). According to these and earlier observations EBV DNA seems to appear in plasma or serum during more severe EBV infections. Our PTLD patients showed increasing amounts of EBV DNA in serum upon progression of the clinical disease; the levels of EBV DNA increased rapidly towards approaching death. Niesters and colleagues (2000) reported very high levels of EBV DNA in plasma in PTLD (mean 540 000 copies/ml), moderately high levels in infectious mononucleosis (mean 6400 copies/ml) and lower levels (mean 440 copies/ml) in transplant recipients without PTLD. Kullberg-Lindh and colleagues (2006) analysed with qPCR serum specimens of pediatric liver transplant recipients; the EBV DNA levels were significantly higher in primary symptomatic infection (mean 65 500 copies/ml) compared with primary asymptomatic infection (mean 3700 copies/ml). Also among the recipients with non-primary EBV infection, the patient with PTLD had higher EBV DNA levels than those without symptoms (5200 copies/ml v.s. 1360 copies/ml) (Kullberg-Lindh et al., 2006).

The sensitivity of qPCR with serum samples seems to be exellent (studies III, IV, V and VI; Orii et al., 2000; Kullberg-Lindh et al., 2006). The specificity is also better with serum; healthy subjects with latent EBV infection have no EBV DNA in serum, and nonsymptomatic immunosuppressed recipients have undetectable or low levels of EBV DNA in serum or plasma (studies III, IV, V and VI; Niesters et al., 2000; Wagner et al., 2000; Brengel-Pesce et al., 2002; Clave et al., 2004; Kullberg-Lindh et al., 2006). Additionally, also stored samples can be studied upon use of serum or plasma. The actual volume of serum (or plasma) needed for qPCR assay of our type is very small (200 µl). Quantification of EBV DNA in plasma or serum is the sample of choice especially in situations where the cell counts are too low for reliable qPCR results,

10.6 TREATMENT OF PTLD

The cornerstone of PTLD treatment is general reduction of immunosuppression. Because of the progressive nature of PTLD, the key to management is prompt or even pre-emptive treatment; with infusions of anti-CD20 antibody, and possibly with donor lymphocytes or EBV-specific cytotoxic T cells (Papadopoulos et al., 1994; Rooney et al., 1998; Ifthikharuddin et al., 2000; Kuehnle et al., 2000; Verschuuren et al., 2002). Early recognition of PTLD is particularly important in the SCT setting, because PTLD in these patients tends to be rapidly progressive (Loren et al., 2003).

10.6.1 REDUCTION OF IMMUNOSUPPRESSION

The effectiveness of immunosupression was described initially by Starzl and colleagues in 1984, and has been substantiated by Tsai and colleagues (2001). Tsai and colleagues (2001) reported an 89% success rate for this strategy for patients who lacked all of the risk factors (organ dysfunction, multiple visceral site involvement and a serum lactate dehydrogenase (LDH) value of >2.5 times the upper limit of normal). So, early action is advisable, before overt PTLD has developed (Cesaro et al., 2004). Even though withdrawal of immunosuppression in PTLD is clearly beneficial, significant risks accompany this mode of treatment. In solid organ transplant (SOT) recipients, graft rejection occurs as a complication in 39% of both responders and nonresponders (Tsai et al., 2001). In patients with life-sustaining organ transplants such as hearts, livers and lungs, reduction in immunosuppression should be moderate and closely monitored, as rejection may be fatal (Loren et al., 2003). In SCT, complications typically manifest as increased risk for GVHD with significant risk for morbidity and mortality. The EBV status does not predict the response to the reduction of immunosuppression (Tsai et al., 2001).

10.6.2 ANTIVIRAL THERAPY

A life-threatening disease, PTLD must be diagnosed and treated early for a favourable clinical outcome. Reduction of immunosuppression remains the cornerstone of PTLD therapy and can result in permanent cure (Loren et al., 2003). Initial attempts to prevent PTLD in the solid-organ transplant population were focused primarily on using antiviral therapies, such as thymidine kinase inhibitors ganciclovir or acyclovir, to eradicate or control EBV for high-risk patients (Loren et al., 2003). These drugs inhibit the replication of other herpes viruses, such as herpes simplex and cytomegalovirus. In vivo, however, they are ineffective against EBV, because EBV survives as an episome outside of the lymphocyte's genome. In addition, these drugs do not eradicate latently infected B cells (Colby et al., 1980; Crumpacker, 1996; Loren et al., 2003). The reports that prophylactic antiviral drugs minimize PTLD risk have been somewhat unconvincing, involving very small numbers of patients in observational studies (Loren et al., 2003). Anecdotal reports of acyclovir or ganciclovir for successful PTLD treatment have not been substantiated (Benkerrou et al., 1993; Loren et al., 2003). However, most of the other therapies have been combined with high-dose antiviral therapy, usually acyclovir. Thus, it is difficult to assess the true utility of antivirals. An interesting approach involves the use of arginine butyrate to induce latent EBV thymidine kinase expression, followed by treatment with ganciclovir (Faller et al., 2001).

The combination of reduction of immunosuppression and antiviral agents (ganciclovir, famciclovir or valacyclovir) has also been used successfully with pediatric liver transplant recipients (Green et al., 1997; Kogan et al., 1999; Holmes et al., 2002). Acyclovir and ganciclovir both demonstrate in vitro activity against the linear (replicating) form of EBV, but neither agent is active against the circular EBV episome in immortalized B cells. However, data from nonrandomized studies suggest that sequential treatment with intravenous ganciclovir followed by oral acyclovir or intravenous ganciclovir alone may be effective (Davis et al., 1995; McDiarmid et al., 1998; Holmes et al., 2002). Interestingly, treatment with CMV IgG has yielded promising results: Holmes and colleagues reported (2002) on five patients with PTLD, who did not respond to the initial interventions, and were subsequently given intravenous CMV IgG. CMV IgG contains in high titers antibodies from pooled adult plasma, and also IgG antibodies against EBV in concentrations similar to those in standard intravenous immunoglobulin products (Holmes et al., 2002). The EBV DNA PCR level fell in all five of these patients during the course of CMV IgG treatment. A similar outcome (reduced EBV DNA PCR levels after reduction of immunosuppression, antiviral therapy, and CMV IgG) has been reported by others (Green et al., 1997 and 1998; Kogan et al., 1999).

10.6.3 EBV-SPECIFIC CYTOTOXIC T CELLS

Heslop together with his colleagues and Rooney with his colleagues demonstrated that PTLD resolved after transfer of EBV specific cytotoxic T lymphocytes (CTLs) grown from donor PBMCs. The method utilized the donor's autologous EBV-immortalized lymphoblastoid B-cell lines (LCLs) cocultured with donor PBMCs in the presence of interleukin-2. LCLs can activate polyclonal EBV-specific CTLs. The adoptive transfer of EBV-specific CTLs grown from donor PBMCs was successful both in prophylaxis and treatment of PTLD in BMT recipients. These EBV-specific CTLs not only persist for long periods in BMT patients and contribute to a memory CTL response but they also proliferate in vivo in the presence of immunosuppressive agents (Heslop et al., 1996; Rooney et al., 1998; Davis and Moss 2004).

A modification of the protocol for growing *ex vivo*-derived EBV-specific CTLs using autologous LCLs was successful in stimulating PBMCs from SOT patient with PTLD (Khanna et al., 1999). Adoptive transfer of EBV-specific CTLs into the patient resulted in regression of tumor burden without autoreactivity or graft rejection. Sherrit and colleagues (2003) demonstrated that EBV-specific CTLs expanded both the magnitude of a memory CTL response and prevented the recurrence of PTLD. Other studies have also successfully used adoptive transfer of autologous EBV-specific CTLs both in prophylaxis and treatment of PTLD in SOT recipients, particularly in early-onset disease (Savaldo et al., 2001; Comoli et al., 2002). One problem with using EBV-specific CTLs is the time required to generate sufficient numbers of cells for infusion. If a patient presents with PTLD without PBMCs being collected previously, it takes approximately 3 months to generate EBV-specific CTLs (Davis et Moss, 2004). Thus alternative treatments such as chemotherapy, surgical removal of tumor, or anti-CD20 monoclonal antibody therapy are often commenced. Ideally, a bank of autologous EBV-specific CTLs should be generated and stored for all SOT patients at high risk of developing PTLD (Davis et Moss, 2004).

As most SOT patients at high risk of developing PTLD are seronegative at the time of transplantation, it is not known how soon post-transplant EBV-specific CTLs can be grown from these patients (Davis et Moss, 2004). The ability to generate autologous EBV-specific CTLs from EBV-seronegative people has been investigated; LCL-stimulated EBV-specific CTLs grown from seronegative adults were $CD3^+CD8^+$ and cytotoxic, but CTLs grown from seronegative children were $CD4^+CD25^+$ and did not recognize autologous LCLs (Savaldo et al., 2002). However, $CD4^+CD25^+$ T cells positively selected from PBMCs stimulated with dendritic cells from these children expanded into EBV-specific CTLs capable of HLA class II-restricted killing (Savaldo et

al., 2002). This method could potentially be used to select for EBV-specific CTL precursor cells in EBV-seronegative patients prior to transplantion, in order to generate an immunotherapy for PTLD.

10.6.4 ALTERNATIVE PTLD THERAPIES FOR SOT PATIENTS

An alternative for the use of autologous EBV-specific CTLs in the treatment of PTLD is the use of partially HLA-matched allogeneic CTLs from an unrelated donor. It is known that EBV-positive individuals with particular HLA types will almost always have EBV-specific CTLs that will respond to particular immunodominant EBV epitopes (Davis et Moss, 2004). For example, HLA B8 individuals will respond to the EBNA 3 epitope FLRGRAYGL, and HLA A2.01 EBV-specific CTLs will recognize the lytic BMLF1 epitope GLCTLVAML (Burrows et al., 1990; Steven et al., 1997). A polyclonal EBV-specific CTL culture from an HLA A2 donor with broad reactivity to a range of lytic and latent EBV proteins should recognize and kill tumor cells in an HLA A2 SOT patient with recipient-origin PTLD (Davis et Moss, 2004). This hypothesis has been tested in a SOT patient with PTLD, and a single infusion of HLA-matched allogeneic CTL resolved the disease without compromising the graft function or inducing autoimmunity (Haque et al., 2001). This study was extended, and complete remission was induced in three of five patients with early localized PTLD, while the two patiens had no response (Haque et al., 2002). Thus, allogeneic HLA-matched EBV-specific CTLs can be used for the treatment of PTLD in patients with common HLA types, when autologous CTLs are not available.

10.6.5 ANTI-CD20 MONOCLONAL ANTIBODY

An emerging therapy for PTLD is the anti-CD20 monoclonal antibody. Typically, four to six doses of anti-CD 20 antibody are administered i.v. weekly, which usually depletes $CD20^+$ B cells for over 12 months (Davis et Moss, 2004). Anti-CD20 antibody was initially utilized to treat chemotherapy-refractory Hodgkin's lymphoma (Keilholz et al., 1999), and phase II studies indicated that it was effective and well tolerated with few side effects (Rehwald et al., 2003; Ekstrand et al., 2003). The overall response was very good; complete remission was observed in 40-60% of Hodgkin's lymphoma patients, and a partial response in most other patients (Davis et Moss, 2004). In one study, the potential for tumor relapse was significant (9/22 patiens), with a median time of only ten months, thus raising questions of long-term efficacy (Ekstrand et al., 2003).

A study involving twelve BMT children with PTLD indicated that only one of 48 infusions of anti-CD20 antibody was associated with a grade II adverse event, and eight children went into complete remission (Faye et al., 2001). Another study tested anti-CD20 antibody on three SOT patients with

PTLD; the outcome was one complete remission, one relapse two months later, and one death from hypogammaglobulinemia-associated aspergillus infection (Verschuuren et al., 2002). The reduction of EBV DNA levels in PBMCs after anti-CD20 treatment is immediate, but this does not always correlate with remission of PTLD (Yang et al., 2000). A combination of anti-CD20 antibody treatment followed by adoptive transfer of EBV-specific CTLs may enhance EBV immunity in PTLD patients, and thus reduce relapse of disease (Davis et Moss, 2004).

10.6.6 LOCAL TREATMENT

When possible, complete surgical excision of localized disease is effective. Localized disease treated with definitive local therapy (surgery or radiation), combined with reduction of immunosuppression, has an excellent prognosis: PTLD-related mortality rates have reported between 0 and 26% (Benkerrou et al., 1993; Davis et al., 1998; Tsai et al., 2001; Loren et al., 2003).

10.6.7 CYTOKINE THERAPY

Attemps to establish a competent immune system to control EBV-related lymphoproliferations with immune modulators such as cytokines with or without immunoglobulins have been made. Case series and case reports have described reponses to interferon-α and interferon-α combined with intravenous IgG (Shapiro et al., 1988; Faro et al., 1996; Davis et al., 1998; Gross et al., 1999; Loren et al., 2003). Interleukin-6, a cytokine that promotes the growth and proliferation of B cells, provides another potential target (Loren et al., 2003). It is difficult to assess the effectiveness of cytokine therapy as most studies have also incorporated concurrent reduction in immunosuppression or antiviral agents. Early reports are encouraging (Shapiro et al., 1988; Benkerrou et al., 1993; Faro et al., 1996; Davis et al., 1998; Haddad et al., 2001).

10.6.8 CYTOTOXIC CHEMOTHERAPY

Chemotherapy has also been used to treat PTLD, generally after the patients have failed to respond to surgical excision with or without reduction of immunosuppression. Regimens are similar to those used for non-Hodgkin's lymphoma, such as CHOP (Loren et al., 2003). While chemotherapy may occasionally provide long-term relapse-free survival, it is accompanied by a high infection and mortality rate (Swinnen et al., 1995; Mamzer-Bruneel et al., 2000; Loren et al., 2003).

10.6.9 CONCLUSION OF PTLD THERAPY

PTLD is an often-fatal complication of both solid-organ and stem cell transplantation. Early diagnosis of PTLD is important and requires high levels of clinical vigilance. Reduction in immunosuppression remains the primary therapy for PTLD and can often result in permanent disease eradication. Further therapies should be tailored to the patient and clinical situation. Treatment with anti-CD20 antibody of SCT recipients seems to be succesful.

10.7 EBV NEGATIVE PTLD – A DIFFERENT ENTITY?

PTLD is usually, but not invariably, associated with EBV. The reported incidence of EBV-negative PTLD varies widely, and it is uncertain whether it is analogous to EBV-PTLD. Leblond and colleagues (1998) reported that PTLD were EBV negative in 34% of patients based on LMP-1 immunostaining, EBER ISH, and in most patients on Southern blot analysis. The EBV-negative PTLDs occurred later than did the EBV positive cases (Nelson et al., 1996; Leblond et al., 1998; Nelson et al., 2000). The EBV-negative cases were monomorphic B-cell PTLDs or polymorphic clonal PTLDs, and the prognosis was very poor (Leblond et al., 1998). Nelson and colleagues (2000) reported that as many as 21% of PTLD patients (17/80) had an EBV-negative PTLD; and 14% of all samples (18/133) were EBV negative. Of the 17 patients with EBV negative PTLD, eight had liver transplants, six kidney transplants, two heart transplant, and one bone marrow. The incidence of EBV negative PTLD seems to be increasing; all but one of the EBV negative PTLDs occurred after 1990 (Nelson et al., 2000). Four patients with EBV-negative PTLD, all with clonal B cells, showed complete remission with mere reduction of immunosuppression (Nelson et al., 2000). Of the nine patients with EBV negative monomorphic PTLD, seven died despite chemotherapy (Nelson et al., 2000). Leblond and colleagues (1998) reported EBV negativity to be an adverse prognostic indicator: 9/11 patients with EBV-negative PTLD died, including seven treated with chemotherapy. The two survivors received combination chemotherapy, and one was disease free at 44 months, while the other was only in partial remission at 12 months. Therefore, EBV-negative PTLDs have distinct features, yet some do respond to decreased immunosuppression, similar to EBV-positive cases (Nelson et al., 2000).

10.8 FURTHER IMPLICATIONS OF EBV qPCR

In conclusion, quantitative PCR for EBV DNA seems to be an exellent approach for diagnosing EBV infections and most importantly PTLD in transplant recipients. Also, cell-free EBV DNA has been detected in the plasma and serum of patients with nasopharyngeal carcinoma (NPC) (Mutirangura et al., 1998; Lo et al., 1999). Lo and colleagues (1999) observed continuously low or undetectable levels of EBV DNA in serum samples among patients who remained in remission. This suggests that quantitation of cell-free EBV DNA may be a valuable tool for monitoring of NPC patients against tumor recurrence. Bortolin and colleagues (2006) followed up a large cohort of patients with undifferentiated carcinoma of nasopharyngeal type (UNCT) in Italy and noticed during follow-up a statistically significant difference of EBV DNA loads between patients with and without clinical relapse. They concluded that EBV DNA reflects the biological activity of UNCT and may be a prognostic factor also in a low-incidence region. However, quantitative EBV PCR is not suitable for a screening test for individuals at high risk for nasopharyngeal carcinoma (Yang et al., 2006).

In EBV-positive lymphomas among immunocompetent patients, release of EBV DNA from tumor cells to plasma could be useful for disease monitoring. Au and colleagues (2004) quantified serially plasma EBV DNA by qPCR in 39 cases of EBV positive (natural killer cell 23, T cell 8, B cell 4, Hodgkin 4) lymphomas. In all cases of EBV-positive lymphomas, EBV DNA was detectable at diagnosis (10^5-10^{10} copies/ml). It paralleled the clinical course, with EBV DNA becoming undetectable at remission and remaining elevated in refractory disease. On multivariate analysis high-level presentation of EBV DNA ($<7.3 \times 10^7$ copies/ml) was significantly associated with an inferior overall survival. Au and colleagues (2004) concluded that in EBV-positive lymphomas, plasma EBV DNA is valuable as a tumor biomarker and for prognostication. Recently, Gandhi and colleagues (2006) reported that EBV-DNA in plasma can be used as a non-invasive biomarker for EBV-positive Hodgkin's lymphoma and serial monitoring of EBV DNA levels could predict response to therapy.

EBV is also associated with pathogenesis of AIDS lymphoma and viral DNA is present within the malignant cells in about half of the patients. Fan and colleagues (2005) analysed by quantitative PCR, EBV-DNA in plasma of all EBER-positive AIDS lymphoma patients, and the viral loads fell rapidly upon initiation of treatment except in two patients with persistent tumor. They concluded

that EBV qPCR is very promising in diagnosis and management of EBV-related lymphomas. Detection of EBV DNA in the cerebrospinal fluid (CSF) is associated with AIDS-related brain lymphoma. Bossolasco et al. (2002) quantified EBV DNA in CSF and plasma from patients with AIDS-related non-Hodgkin's lymphoma (NHL). High CSF EBV DNA levels were found in HIV-associated brain lymphomas. Bossolasco and colleagues (2006) have recently quantified EBV DNA load in CSF of patients with HIV-related brain lymphomas: they noticed the EBV loads to be significantly lower during and after ganciclovir treatment indicating that this drug might be useful in management of HIV-related CNS lymphoma.

10.9 EBV INFECTIONS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION (VI)

Our final goal was to determine the number and clinical significance of EBV infections, and their relation with EBV-associated PTLD in a large cohort on unselected SCT recipients. Of the 406 SCT performed in Helsinki during 1988-1999, 5479 consecutive samples of serum were retrospectively studied by EBV qPCR.

Altogether, EBV infections after stem cell transplantation were relatively common; 14% of our patients had detectable EBV-DNA in at least one serum sample. This is in concordance with others' observations; Cesaro and colleagues (2004) reported EBV reactivation (as determined by increasing viral load by qPCR) in 53% of pediatric allo-SCT recipients. The higher rate of EBV DNA positivity in that study may have been be due to two (or more) reasons; the patients were children (median age 9.6y), and the sample material was blood mononuclear cells. The only risk factor significantly associated with EBV reactivation was ATG. Earlier, van Esser and colleagues (2001) analysed plasma samples from adult SCT recipients using qPCR and reported an indicidence of EBV reactivation of 28%. In that study, none of the unmanipulated SCT recipients developed PTLD. Clave and colleagues (2004) detected EBV DNA in peripheral blood mononuclear cells of 50% (28/56) of allo-SCT recipients. Plasma EBV DNA was detected in 16% (9/56) of recipients, who also displayed the highest cellular viral loads (Clave et al., 2004).

In the present retrospective study 22 (5.4%) recipients showed high copy numbers (>50 000) of EBV DNA. The EBV-DNA levels of these patients rose progressively, with the peak levels occurring shortly before death, or with the counts high already in the first positive sera. The prognosis of these patients was sinister; all cases were fatal. Cesaro and colleagues (2004) observed in 35% of their pediatric SCT patients EBV DNA counts of ≥ 300 gekv/100 000 PBMC; the

immunosuppression was electively reduced in patients who exceeded this cut-off. In that study, 16% of all the recipients showed clinical symptoms of EBV infection (i.e. fever, lymphadenopathy, hypertrophy of Waldeyer's ring), and PTLD was diagnosed in one (1%). However, no deaths caused by PTLD were observed among patients with EBV reactivation. Wagner and colleagues (2004) studied by qPCR the PBMC of SCT recipients, of whom 11% (9/85) showed single episodes of a high EBV DNA level (>4000 EBV copies/μg PBMC DNA) and 19% (16/85) showed two or more episodes. 9.4% (8/85) of all recipients developed symptomatic PTLD and were successfully treated with EBV-specific cytotoxic T cells (two patients), anti-CD20 antibody (five patients) or both (one patient). In all the eight patients clinical symptoms associated with increased EBV load disappeared. With EBV qPCR screening Wagner and colleagues (2004) recommended prompt rather than pre-emptive treatment, following the onset of PTLD symptoms.

Of our 22 (5.4%) recipients with severe EBV infection, all except one showed EBV DNA very early, within two months (median) after transplantation. In one patient however, the EBV DNA appeared after DLI, at ten months post-transplantation. All the patients with excessive amounts of EBV DNA had an aggressive type of disease: the time from the first EBV-DNA-containing sample to death was 18 days (median). These observations are in concordance with our earlier studies (study III). In addition, 16 (3.9%) stem cell recipients showed a low-level EBV-DNA in the last sample preceding death. Five of these patients showed in post-mortem study histologically confirmed fatal PTLD; such last samples of these five patients were taken 80 days (median) preceding death.

By contrast, 19 (4.7%) patients showed only transient EBV-DNA positivity, during the first three months (median) after transplantation. The EBV DNA copy numbers of this group remained significantly lower compared to the patients with fatal EBV infection; only one of these patients had a relatively high level (36 200 copies) of EBV DNA. Importantly, the EBV DNA of all these patients disappeared without clinical intervention, and none developed EBV PTLD during follow-up. Our data thereby show that low-level EBV DNA positivity in serum occurs frequently and may resolve spontaneously.

For identification of the patients with severe EBV infection, we created an algorithm which distinguished all the patients with a fatal EBV infection from those with a transient EBV reactivation. In the former patients unlike in the latter, with the EBV DNA levels rising rapidly, the

mean value of the derivatives of the log-transformed DNA values exceeded the cut-off value of 3.6, cautioning against life-threatening EBV infection.

In conclusion, low-level EBV-DNA positivity in serum occurs relatively frequently after stem-cell transplantation and may subside without specific treatment. However, high molecular copy numbers (>50 000) predict the development of PTLD very strongly, are not spontaneously reversible, and warrant immediate treatment. Based on the EBV qPCR data, we furthermore created an algorithm that distinguished the patients with severe EBV infection from those with transient infection.

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